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The remaining obstacles to achieving reliable therapeutic effects by neurotransplantation for Parkinson's disease (PD) are 1) poor survival of grafted fetal neurons and 2) insufficient axonal outgrowth and functional recovery.

We carried out experiments aimed at by short-term inhibition by pre-treatment of fetal cells with pharmacological inhibitors of caspases. In our second objective of enhancing axonal growth leading to optimal functional recovery by neuronal transplants, we employed transgenic bcl-2 overexpressing donor cells and similar molecules influencing the growth of axons in the fetal and adult CNS. Use of such molecules could significantly improve neural transplantation and related regenerative therapies. Our results indicate that hubcl-2 expression can enhance dopaminergic axonal outgrowth in vivo. Immunophilin ligands and GDNF can also generate increased axonal elongation and branching of dopamine neurons.

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INTRODUCTION:

The remaining obstacles to achieving reliable therapeutic effects by neurotransplantation for Parkinson's disease (PD) are 1) poor survival of grafted fetal neurons and 2) insufficient axonal outgrowth and functional recovery.

While about 50% of cells born in the CNS will die naturally, up to 99% of fetal cells die after neural transplantation, which suggests that a large fraction of this cell death is preventable. Activation of caspases is one of the final steps before a neuron is committed to die by apoptosis. Since fetal development of neuronal grafts in many ways mimics normal development it is very likely that the caspases play a major role in the control of cell death in transplants. The structural brain repair experiments proposed will use fetal DA cells obtained from ventral mesencephalon (VM) of transgenic mice. A: In our first objective of preventing neuronal apoptosis, we will compare long-term or permanent inhibition of apoptotic agents by using donor cells from knock-out (ko) mice (ko: caspase-1, -3 and JNK3), to short-term inhibition by pre-treatment of fetal cells with pharmacological inhibitors of caspases. B: Our second objective of enhancing axonal growth leading to optimal functional recovery by neuronal transplants, employs transgenic bcl-2 overexpressing donor cells. Evidence now exists that the very potent positive effects by the bcl-2 molecule on axonal outgrowth are distinct from its anti-apoptotic effect. We hypothesize that bcl-2 and similar molecules influence the growth of axons in the fetal and adult CNS, and that controlling such molecules could significantly improve neural transplantation and related regenerative therapies.

This basic research can be translated clinically in neurosurgical transplantation therapy for Parkinson's disease patients, for example by similar transgenic porcine donor cell modifications. In summary, by using donor cell modification of molecules involved in apoptosis and axonal growth, these experiments provide insights and therapeutic strategies for preventing neuronal death and enhancing axonal outgrowth.

BODY:

STATEMENT OF WORK

We describe below the research accomplishments associated with the approved Statement of Work, which is copied here in bold. The publications and figures referenced are attached in the Appendix.

STEPS 1.1-1.2 Reduction of apoptotic cell death leading to greater cell survival in dopaminergic neuronal grafts can be achieved by permanent deletion of apoptotic effectors such as Caspase 3/CPP32 and JNK3 in transplanted neurons:

In year 1-2, we will start the transplantation experiments of embryonic day 14 (E14) ventral mesencephalon (VM) cells from CPP32 and JNK3 deficient (knock out) mice and wild type (wt) litter mate controls into the striatum of adult parkinson model rats; obtained by neurotoxic lesion using 6-OHDA. Functional recovery will be determined by the decrease observed in amphetamine-induced rotational asymmetry at various time points (4 to 12 weeks) after transplant. Post-mortem microscopic analyses, using markers for tyrosine hydroxylase (TH) and aldehyde dehydrogenase (AHD), will reveal the extent of the increase in surviving TH and TH/AHD. The knock-out donor cells for the dominant Caspase 3 and the stress-induced JNK3 will provide separate evidence for the apoptotic pathways that are involved in neural transplant cell death. These results are compared to those obtained after incubating the wild-type donor tissue short-term (1 hour) with selective and non-specific synthetic caspase inhibitors *prior* to transplantation (since these molecules z-DEVD.CHO/fmk; and BAF (Bocaspartyl(OMe)-fluoromethylketone) have poor penetration across the blood-brain barrier).

We started the transplantation experiments of embryonic day 13-14 ventral mesencephalon from CPP32 and JNK3 knock-out mice and their counterpart wild-type. We discovered immediate problems with obtaining a sufficient number of CPP32 knock-out fetuses because the unexpected abnormal formation of the ventral mesencephalon, including dopaminergic neurons. For this reason, we abandoned the knock-out part of the CPP32 experiment but did go ahead with using the synthetic caspase inhibitors in various transplant experiments (see abstract, Cicchetti et al. 2000) using BAF (Bocaspartyl(OMe)-fluoromethylketone) for the pretreatment of these cells. These experiments are reported below and we are continuing in the second year with an adapted methodology for preventing early apoptotic cell death in these systems.

STEPS 2.1: Transplantation of dopaminergic neurons permanently expressing human bcl-2 leads to enhanced and long-distance axonal outgrowth and improved function of neuronal transplants used for Parkinson's disease:

STEP 2.1.1. Normally, transplantation of ventral mesencephalon (VM) cells to a single site in the striatum will only result in fetal axonal penetration of the tissue immediately surrounding the graft. In year 1-2, we will carry out a conclusive series of experiments with transplant donor tissue of E14 VM from bcl-2 overexpressing mice or wild type (wt) control mice to the striatum of parkinson model rats, with prior neurotoxic 6-OHDA damage. We will investigate functional recovery by amphetamine induced rotation over an 8 to 10 week period. At post-mortem, by immunohistochemistry for TH, AHD and human bcl-2 (hubcl-2) we will determine axonal outgrowth and the extent of axonal outgrowth into the surrounding host striatum.

In step 2.1, we have carried out the transplantation of hubcl-2 cells. This has led to one manuscript in preparation (Appendix). We have shown that bcl-2 overexpressing mice (approximately 50% of the cells) have some enhanced outgrowth of transplanted dopaminergic neurons into the striatal system. These transgenic experiments have been difficult to carry out because of problems with breeder pairs and the difficulty of obtaining a sufficient number of pregnant mice. Changing to Jackson Laboratories acquisition of the transgenic mice should lead to easier access.

STEP 2.1.2. Prior work has shown that transplantation of wt VM cells to the substantia nigra does *not* result in long distance growth to the target host striatum. Continuous expression of bcl-2 will allow axons from the bcl-2 transgenic cells to reach distant host targets. We will therefore transplant E14 VM from bcl-2 overexpressing mice to the substantia nigra of the parkinson model rats. Again, we will investigate functional recovery through amphetamine induced rotation over an 8 to 10 week period. By retrograde tracing combined with immunohistochemistry for TH, AHD and specific staining for human bcl-2; we will determine axonal outgrowth and completeness of reconnection of the damaged neuronal circuitry.

In step 2.1.2., we have planned and transplanted bcl-2 overexpressing cells into the substantia nigra for long-distance growth. Early experiments showed surprising limited survival of both wild-type and transgenic mice in that location and we are trying to work out parameters and differences to achieve more survival in the substantia nigra graft location.

STEP 2.1.3. (Deleted by Reviewers)

STEP 2.1.4 The time course of bcl-2 expression is correlated with axonal extension from neurons in primary cell culture, similar to expression and outgrowth patterns seen in

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normal development. Bcl-2 overexpressing cells will continue to extend axons after 8 days in vitro (DIV) when the axonal outgrowth from normal cells has stopped. In this step, we will investigate the expression of bcl-2 in cultured VM neurons from wt controls and bcl-2 overexpressing mice and measure axonal growth in both, following double immunohistochemical labeling for TH, AHD, mouse- and hubcl-2. The cultures will be fixed at various time points from 2 to 12 DIV. In primary wt cell cultures, we will also examine if cells from different parts of the CNS with different rate of maturation will have variable but coinciding patterns of bcl-2 expression and axonal outgrowth. The expression of bcl-2 in VM neuronal cultures will then be compared to its expression in neuronal cultures derived from other parts of the developing CNS such as basal forebrain (septum), cerebellum and lateral ganglionic eminence, from bcl-2 overexpressors and wt controls and stained with bcl-2 and markers for specific subpopulations of these neuronal regions. These experiments will provide axonal growth data from different anatomical regions and specific neuronal phenotypes; with different rate of maturation will and corresponding bcl-2 expression. These data will be be applied to neural transplantation surgery requiring extensive or long-distance axonal growth for therapeutic repair or regeneration.

We have carried out cell culture studies for expression of various outgrowths of the dopaminergic neurons and have stained for human bcl-2 (see figure). We have examined the expression of the rodent systems for bcl-2 overexpression (see article, appended) and are currently investigating other cell types and species.

STEP 2.2. The cell cycle regulating molecules p21 and p53 bring the neuron to a terminal differentiation stage. Bcl-2 appears to be involved in the signals to block these molecules, thereby keeping the neuron in an elongation stage. In our final experiments of this series, bcl-2 overexpressing cells will be used as tools to investigate *other* molecules that act on axonal growth. The expression of the molecules FKBP12, calcineurin, p21, and p53, will be investigated in primary cell cultures of dopaminergic neurons from bcl-2 transgenic or wt mice, via western blot and immunostaining. This final step will lead us to additional molecules; relevant for future approaches for enhanced axonal growth and regeneration in therapies for patients with sustained neuronal damage or degeneration.

We have carried out cell culture experiments to start examining other growth-related molecules such as FKBP12 and calcineurin in primary dopaminergic cultures to see how this molecule may change outgrowth we have found data that support a mechanistic role of phosphorylation signals in the axonal elongation stage (see manuscript, Costantini and Isacson, 2000, which will be referred to and referenced appropriately to this project).

KEY RESEARCH ACCOMPLISHMENTS:

- Pharmacological caspase inhibitors can prevent some apoptotic cell death in conjunction with neurotrophic factors.
- Transgenic donor cells with caspase 3 and JNK3 deficiency may not develop appropriate ventral mesencephalic substantia nigra dopaminergic regions and therefore not be useful for transplantation.
- Overexpression of the bcl-2 gene in dopaminergic neurons can enhance their axonal growth capacity. After cell transplantation of mice hubcl-2 expressing fetal dopaminergic neurons to 6-OHDA lesioned rat striatum, an enhanced axonal outgrowth was seen compared to control wild-type cells.
- Axonal elongation and branching in dopaminergic neurons may be controlled by different intercellular signalling involving at times FKBP-12, calcineurin, p21 and p53.

REPORTABLE OUTCOMES:

Manuscripts:

1. Holm, K. and Isacson, O. (1999) Factors intrinsic to the neuron can induce and maintain the ability for neurite outgrowth: a role for bcl-2? Trends Neurosci. 22, 269-273.

2. Boonman, Z. and Isacson, O. (1999) Apoptosis in neuronal development and transplantation: role of caspases and trophic factors. Exp. Neurol. 156, 1-15.

3. Costantini, L.C. and Isacson, O.(1999) Dopamine neuron grafts: development and molecular biology. In: Dopamine Neuron Development, U. di Porzio, R. Pernas-Alonso and C. Perone-Capano, eds., R.G. Landes Company, Georgetown, pp. 123-137.

4. Isacson, O. and Sladek, J. (1999) Cellular and Molecular Treatments of Neurological Diseases. Exp. Neurol. 159, 1-3.

5. Kang, U.J. and Isacson, O. (2000) The Potential of Gene Therapy for Treatment of Parkinson's Disease. In: Principles of Surgery for Parkinson's Disease and Movement Disorders, Krauss, K., Jankovic, J., Grossman, R. eds. Lippincott-Raven, in press.

6. Costantini, L.C., Isacson, O. (2000) Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons. Exp. Neurol. 164, 60-70.

7. Holm, K.H., Cicchetti, F., Bjorklund, L., Boonman, Z., Tandon, P., Costantini, L.C., Deacon, T.W. , Chen, D.F., Isacson, O. Enhanced axonal growth from hubcl2 transgenic mouse dopamine neurons transplanted to the adult rat striatum. Manuscript in preparation.

Abstracts:

1. L.C. Costantini, D. Cole, O. Isacson Neurophilin ligand enhances reinnervation of host striatum from fetal dopamine transplants. American Society for Neural Transplantation and Repair, 2000.

2. F. Cicchetti, L. Costantini, A. Moore, R. Belizaire, W. Burton, W. Fodor & O. Isacson. Combined Apoptosis and Complement Inhibitors Improve Porcine Neural Xenotransplant Survival in the Rat Brain. American Society for Neural Transplantation and Repair, 2000.

3. O. Isacson. Neural cell transplantation in neurodegenerative diseases. XVIII Intl. Congress of the Transplantation Society, Rome, 2000.

4. O. Isacson. Development of neuronal repair and reconstruction strategies against neurodegenerative disease. Intl. Workshop on Stem Cell Biology and Cellular Molecular Treatment, Tokyo, 2000.

5. O. Isacson, Primary Neuronal Cell Transplantation for Parkinson's Disease, The Cell Transplant Society, Montreux, Switzerland, Mar. 21-24, 1999.

6. L.C. Constantini, D. Cole, O. Isacson. Neurophilin ligands: neurotrophic effects in models of Parkinson's disease. American Society for Neural Transplantation, 1999.

7. O. Isacson. Dopamine neuron transplantation: pharmacological and behavioral aspects. Behavioral Pharmacology Meeting, Sept. 1-4, 1999.

8. O. Isacson. Neural Transplantation in Neurodegenerative Diseases. Year of the Brain Intl. Symp. Oct. 1-3, 1999.

Presentations:

1999 Cornell Medical School/New York Hospital "Developing nerve cells against neurodegeneration" (grand rounds & lecture)
1999 Montreux, Switzerland, The International Cell Transplant Society, "Primary neuronal cell transplantation for Parkinson's disease (lecture)
1999 Keystone Symposia, "Neural xenotransplantation for neurodegenerative disease" (lecture)
1999 Dalhousie University, Halifax, Clinical Neuroscience (rounds) and Dept. of Anatomy and Neurobiology (lecture)
1999 University of Pittsburgh Medical Center, Dept. of Pathology (lecture)
1999 University of Rochester, Experimental Therapeutics Workshop (lecture) and Neurology Grand Rounds
1999 Vancouver, BC, XIIIth Intl. Congress on Parkinson's Disease (lecture)
1999 Odense, Denmark, 7th Intl. Neural Transplantation Meeting (lecture)
1999 Boston, European Behavioral Pharmacology Society and Behavioral Pharmacology Society Conference (lecture)
1999 Austrian Parkinson Society, Vienna (lecture)
1999 Bonn, Intl. Neuroscience Symposium "Molecular Basis of CNS Disorders" (lecture)
1999 London, The Novartis Foundation "Neural Transplantation in Neurodegenerative Disease"
1999 Miami, 6th National Parkinson's Foundation Intl. Symposium on Parkinson's Research (lecture)
2000 Louisville, "The Neuroscience of Developing Cell Therapies for Parkinson's Disease" (lecture)
2000 Zurich, Intl. Study Group on the Pharmacology of Memory, (lecture)
2000 Tokyo, Intl. Workshop: Stem Cell Biology & Cellular Molecular Treatment (lecture)
2000 Il Ciocco, Italy, Gordon Research Conference (lecture)
2000 Rome, Intl. Cong. of the Transplantation Society (lecture)
2000 Turin, Italy, Cellular & Molecular Mechanisms of Brain Repair (lecture)

CONCLUSIONS:

Our data in this project indicate that the bcl-2 molecule can modify the growth state of the axon of the neuron. The experiments have demonstrated that the implantation of hubcl-2 overexpressing mouse neurons into the rat dopamine denervated striatum can lead to a slight improvement in donor axonal outgrowth. Other experiments indicate that neurotrophic factors such as GDNF and immunophilin ligands also can modify axonal elongation and branching patterns at least of cultured dopaminergic neurons. The effort to derive a better treatment for patients and U.S. service personnel with Parkinson's disease is supported by these cell transplantation studies. The transgenic modification of dopaminergic neurons and the successful transplantation of a number of these dopamine producing cells may lead to improved functional recovery in surgical transplantation therapies.

APPENDICES:

Figures and Figure Legends

Publications:

Manuscripts:

1. Holm, K. and Isacson, O. (1999) Factors intrinsic to the neuron can induce and maintain the ability for neurite outgrowth: a role for bcl-2? Trends Neurosci. 22, 269-273.

2. Boonman, Z. and Isacson, O. (1999) Apoptosis in neuronal development and transplantation: role of caspases and trophic factors. Exp. Neurol. 156, 1-15.

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7. Holm, K.H., Cicchetti, F., Bjorklund, L., Boonman, Z., Tandon, P., Costantini, L.C., Deacon, T.W. , Chen, D.F., Isacson, O. Enhanced axonal growth from hubcl2 transgenic mouse dopamine neurons transplanted to the adult rat striatum. Manuscript in preparation.

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Curriculum Vitae: Dr. Ole Isacson

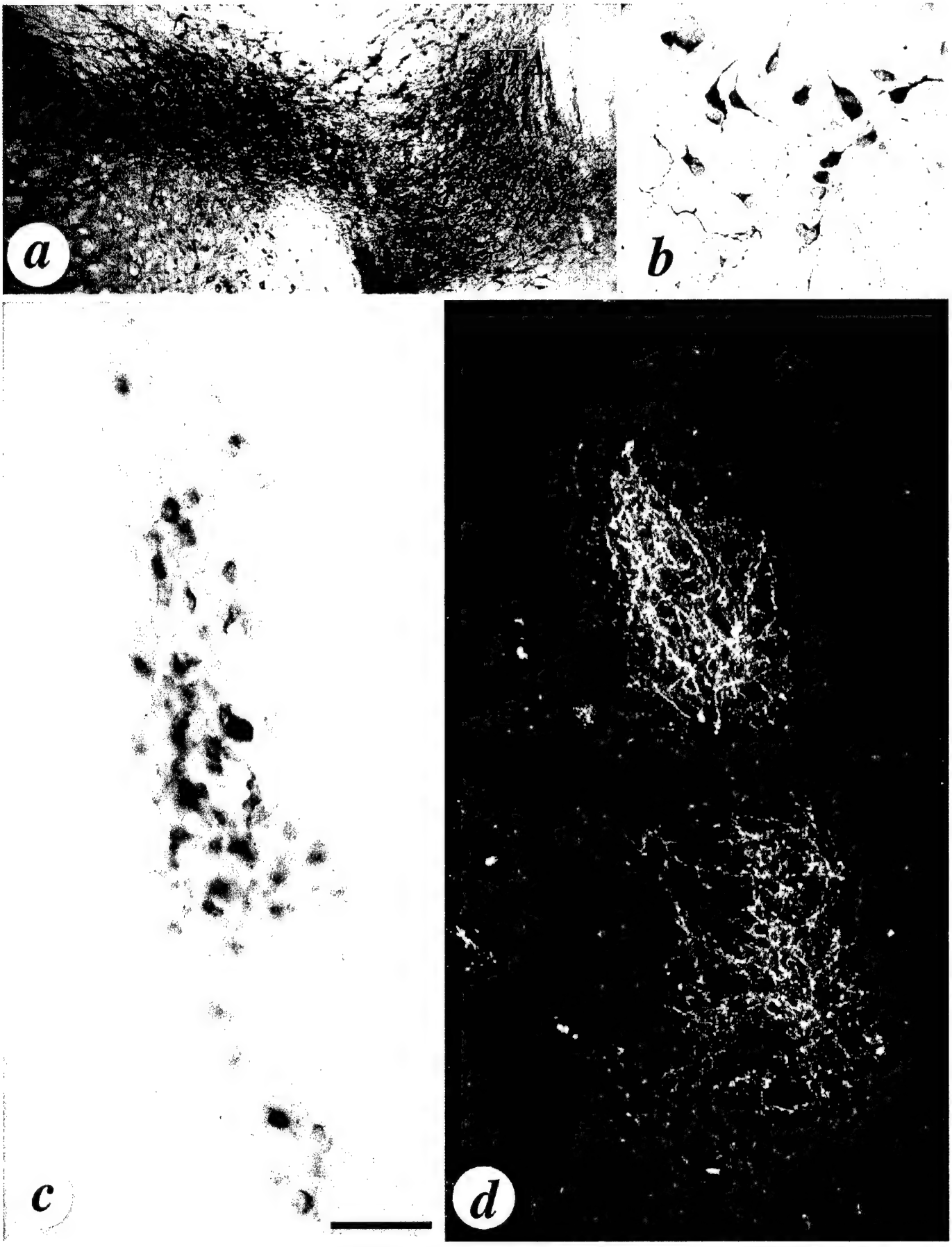
Figure Legends

Figure 1:

HuBcl2 staining of the adult transgenic mouse brain (a), VM cell culture (b), xenografts of E14 VM cell suspension from huBcl2 transgenic mice into the substantia nigra of the rats (c-d). Hublc2 positive (c) and TH positive (d) neurons can be identified in the graft.

Figure 2:

The distance and the number of TH positive fiber crossings in striatal grafts of VM cells from wild type and huBcl2 transgenic mice.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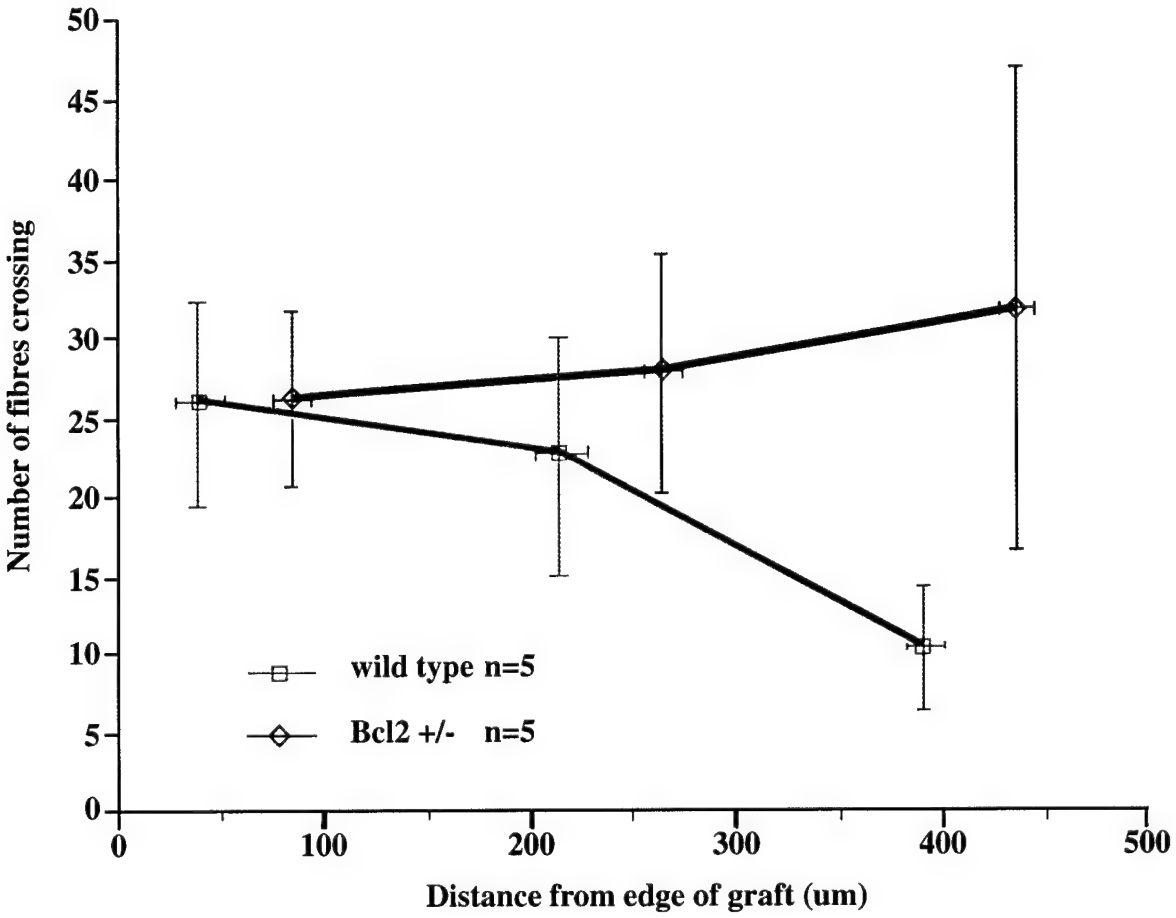
Number of fibres crossing
50
45
40
35
30
25
20
15
10
5
0
0
100
200
300
400
500
Distance from edge of graft (um)
wild type n=5
Bcl2 +/- n=5

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Factors intrinsic to the neuron can induce and maintain its ability to promote axonal outgrowth: a role for BCL2?

Karin Holm and Ole Isacson

The adult CNS provides a poor environment for axonal growth and regeneration. The question of to what extent the loss of axonal growth occurring as the brain matures is dependent on factors intrinsic or extrinsic to the growing neuron is still unanswered. Examination of axonal growth from neural transplants provides insight into the roles of growth factors, inhibitory molecules, growth-promoting substrates and the differences between CNS and PNS environments in the regulation of neurite extension. The data that imply a role for BCL2 and related molecules in such processes are reviewed in this article, which analyzes the factors intrinsic to the neuron that control its capacity for axonal growth.

Trends Neurosci. (1999) 22, 269–273

IMPLANTATION OF NEURONS into the brain provides an *in vivo* assay for measuring permissive and non-permissive axonal-growth substrates, as well as differences in the capacity of neurons to extend their axons. In a recent experiment Davies and colleagues[1] asked the question of whether, in the absence of glial scarring, adult white matter can be growth permissive for axons from an adult neuron and, moreover, whether local production of chondroitin sulfate in the host will decrease the ability of grafted neurons to extend their axons. Using a microtransplantation technique to minimize scarring, they reported rapid axonal extension by grafted adult dorsal-root-ganglion neurons (DRG) for long distances in the host white matter. Grafts with no outgrowth were surrounded by a border of chondroitin sulfate, whereas no increase in proteoglycans was detected in grafts that extended their axons (see Fig. 1A,B). The researchers' interpretation of these data is that the adult CNS environment can still be growth supporting for adult neurons. However, the adult PNS (which contains DRG neurons) normally retains the ability to regenerate axons and, thus, raises the question of whether DRG neurons might also possess intracellular properties that allow them to extend axons for long distances. An alternative interpretation, therefore, is that intrinsic properties of the transplanted PNS neurons, like fetal CNS neurons, can overcome the growth inhibition[4] by the adult CNS environment. In fact, in an experiment in 1977, Björklund and Stenevi[2] transplanted adult sympathetic-ganglion neurons to a cavity in the septo–hippocampal junction of bilaterally sympathectomized adult rats (Fig. 1A,C). They demonstrated that axonal growth was produced by these transplanted nonadrenergic PNS neurons, which extensively reinnervated the host hippocampus across the necrosis of the lesion, and that continuous axonal extension occurred up to three months after transplantation. Axons grew for more than 4 mm into the hippocampus. As spontaneous regeneration by the host-brain noradrenergic axons was prevented by toxic lesion of nonadrenergic neurons located in the locus coeruleus, these experiments demonstrate that PNS neurons have the ability to extend axons for considerable distances in the adult CNS. Given that PNS neurons and some CNS neuronal phenotypes, such as nonadrenergic and cholinergic[2,5], retain their ability to regenerate throughout adulthood, there must be molecules and intrinsic processes that allow such confirmed axonal growth. These intrinsic properties might involve the same growth-promoting molecules that are active during the short time when embryonic transplanted CNS neurons are able to extend axons, despite the inhibitory properties of the adult CNS (Ref. 4).

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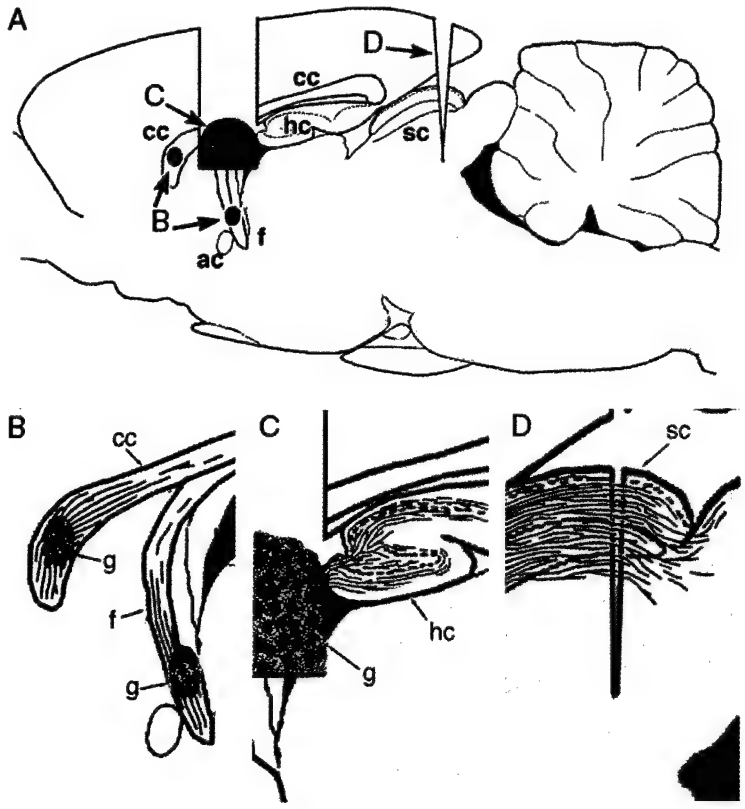

Fig. 1. In vivo *paradigms that illustrate conditions and factors involved in maintaining capacity for long-distance axonal growth in the adult brain.* *By transplanting adult dorsal-root ganglia (DRG) to the white-matter tracts of adult rats [(see red arrows in* **(A)***], Davies* et al.[1] *demonstrated that long-distance axonal growth occurred from the grafted adult DRG neurons into the adult host, unless excessive proteoglycan-rich barriers prevented axonal penetration. The placements of the grafts (red) are illustrated in* (A) *(the upper sagittal view of the rat brain) and in more detail in* **(B)**. *The axons growing within the white-matter tracts are detailed in* (B). *In an earlier but related experimental paradigm* **(C)**, *Björklund and Stenevi*[2] *managed to transplant adult superior cervical ganglia (green) to the site of an adult fimbria-fornix transection in rats. Axons from the adult noradrenergic neurons (green) penetrated the adult dorsal hippocampus (hc) and these axons grew for several millimeters into the adult hippocampus. A third example* **(D)** *of a capacity for axonal growth into CNS territory, which is not normally permissive to penetration, is provided by the experiments of Chen* et al.[3] *In mice that overexpress* Bcl2, *at an age when axonal regeneration in the retino–tectal system is normally abortive (postnatal day 4), these authors demonstrated optic-tract axonal regrowth [dark-blue fibers in* (D)*] through a transected area into the tectum by postnatal day 10. The three* in vivo *examples of regenerative or regeneration-like growth all illustrate situations in which factors intrinsic to neurons can extend the capacity for axonal growth into relatively non-permissive adult neural substrates. Abbreviations: ac, anterior commissure; cc, corpus callosum; f, fornix; g, grafted neurons; hc, hippocampus; sc, superior colliculus.*

In normal development, axonal growth occurs in spurts that are interrupted by growth-cone collapse[6–8]. It appears that molecular gradients of attractive and inhibitory cues provide axonal orientation by inducing asymmetric growth-cone collapse and extension[6]. Axons from transplanted fetal neurons can grow extensively in neonatal host brain, but less so adult host brain[9,10]. Therefore, the adult CNS environment is likely to cause more frequent growth-cone collapse of transplanted neurons, which increases the time needed for the axon to reach its target. Most transplanted neuronal phenotypes only persist in a growing state for a limited time. In fetal allotransplantation to the adult host brain, this time is usually too short for the axon to reach distant targets. Xenotransplantation experiments that use donor species with slower embryonic development than the host, for example, pig[11] or human[12], transplanted to adult rats, show that the longer time window for outgrowth in this paradigm allows the axons to reach long-distance targets. One frequently used explanation for this is that axons from the xenotransplants could be less responsive to growth-inhibiting molecules produced in the host brain. This is certainly possible, but less likely as we have preliminary observations that the growth from xenotransplants in the adult is relatively impeded compared with growth in developing postnatal-day 7 (P7) brain[10], which indicates that growth inhibition seen in allotransplants is also present in xenotransplants. Also, mouse fetal tissue that is xenotransplanted to the rat striatum does not show the increase in axonal growth relative to rat donor tissue that is seen when human tissue is xenotransplanted into the rat[10].

Another reason for the ability of embryonic neurons to extend axons in the adult brain could be that they have yet to produce receptors for certain growth-inhibiting factors. There is, in fact, partial evidence of upregulation of the synthesis of these receptors in developing neurons around the time of myelination[13]. An intrinsic program for axonal outgrowth would, thus, be correlated with a low responsiveness to inhibitory signals from the environment. As the neuron matures, the increased production of the receptors for growth inhibitors would retard its growth. The ability for long-distance axonal growth from xenotransplants could, therefore, be due to the greater length of time that elapses before these receptors are produced, coupled to the delay while certain growth-promoting molecules become active within the neuron.

Fetal neuronal transplantation as a model system: factors extrinsic to the neuron that might be involved in regulation of axonal outgrowth

The adult CNS is a relatively poor environment for axonal growth and regeneration. Nevertheless, fetal neurons are capable of extending axons to some degree, even in the adult brain, especially when transplanted to their axonal target zones. For example, fetal rat ventral-mesencephalic (VM) cells that are allotransplanted to the adult striatum of an animal model of Parkinson's disease will form connections and create behavioral recovery in the recipients[14]. However, in most cases, histological analysis shows that only a part of the target, that which immediately surrounds the graft, is innervated using this transplantation method. Conversely, when transplanted to neonatal brain, these fetal neurons migrate into the surrounding brain and extend axons for long distances[9,10]. Interestingly, the host age at which homotopically transplanted embryonic VM neurons fail to extend axons for a long distance to the striatum appears to be between P8 and P20.

What are the factors involved in this transition of the host environment from one that is permissive to growth to one that inhibits growth? High levels of growth factors are present in the brain during development and correlate in time and location with enhanced growth of specific groups of neurons. For example, the gene encoding NGF is highly expressed in the hippocampus and cortex during development[15], and likewise the gene encoding glial-derived neurotrophic factor (GDNF) is expressed in the developing striatum[16]. There are several studies that show improved innervation of tissue surrounding the graft

when the graft is exposed to growth factors. Granholm and colleagues showed more extensive innervation from dopaminergic cells transplanted to the striatum after pretreatment of the fetal tissue with GDNF (Ref. 17). In addition to improved innervation of nearby target tissue, long-distance growth has also been achieved using growth factors. For example, Wang *et al.*[18] created a track of GDNF along which axons from grafted dopaminergic neurons placed in the substantia nigra (SN) could grow to the target striatum. Furthermore, productions of growth factors could be the cause of more-extensive or long-distance axonal outgrowth from grafted neurons in a number of experiments that use co-transplantation of embryonic tissue. Transplantation of a mixture of fetal VM and striatal cells into adult 6-hydroxy-dopamine-lesioned striatum results in a larger area of dense innervation surrounding the graft[19]. When transplanting embryonic VM cells to the lesioned SN and creating a bridge of embryonic cortical tissue along an oblique needle track from the SN to the striatum, Dunnett and colleagues[20] showed that dopaminergic fibers from the graft could grow along the bridge graft all the way to the striatum. Mendez and colleagues[21] transplanted fetal VM cells simultaneously to the SN and the striatum of adult rats and, surprisingly, growth of dopaminergic axons from the graft in the SN to the VM graft in the striatum was observed, using retrograde labeling with flourogold. As a control, fetal cortical neurons transplanted into the striatum did not cause the homotopically placed dopaminergic neurons to grow to the striatum. A possible explanation for this long-distance axonal growth could be the release of appropriate trophic factors by fetal cells, which thereby stimulate long-distance directional growth of axons, as appropriate axon orienting (tropic) factors are still provided by the adult CNS environment[11].

A number of factors and conditions that inhibit growing neurites have been described, many of which are present in the adult CNS (Ref. 4). Blocking these factors is one approach that can be used to make the adult CNS more permissive for axonal growth and regeneration. Several experiments that used antibodies to growth-inhibiting proteins, such as myelin-associated neurite-growth inhibitors, to treat lesioned axons in the CNS, demonstrate a moderate increase in regeneration Thus, it seems that these proteins are, to some extent, involved in the inhibition of axonal growth in the CNS. Inhibitors of neurite growth and the application of antibodies directed to these inhibitors are reviewed by Schwab[4]. The growth-inhibiting properties of the adult CNS surroundings are not seen in the PNS. Instead, the PNS environment usually provides excellent conditions for regeneration and axonal growth. For example, peripheral myelin has been shown to promote axonal growth, even for CNS neurons[22]. In early transplantation experiments, Aguayo *et al.*[23] used grafted sciatic nerve to connect dopaminergic grafts in the SN to their striatal target. They observed remarkable long-distance growth of dopaminergic fibers through the whole length of the sciatic graft (outside the skull and back into the striatum). In summary, these studies demonstrate the importance of factors present in the surrounding CNS for the ability of neurons to extend and regenerate axons (Table 1).

TABLE 1. Factors and conditions that are involved in axonal outgrowth mainly as studied in transplantation experiments

	Growth promoting	Growth inhibiting
Extrinsic	Fetal host[9] Fetal tissue at target[19,21] Minimal scarring from transplantation[1] Peripheral environment[22,23] Denervated target area[24] Growth-factor stimulation[17,18]	Adult host[10] Proteoglycans (chondroitin sulfate) surrounding the transplant[1] Myelin-associated growth inhibitors[4] Intact target area[24]
Intrinsic	BCL2 (Refs 24,25) Factors present during certain time of fetal development[11,12] Factors in fetal and adult PNS neurons[1,2]	Receptors for growth inhibitors[13] Inhibitory factors or lack of growth-promoting factors in adult CNS neurons[26]

Undoubtedly, the CNS becomes less permissive for axonal growth as the brain matures. Nevertheless, as already described, fetal neurons transplanted to the adult CNS have the ability to extend axons in gray and white matter. What are the factors involved in this ability of the fetal neuron to overcome the growth inhibition by the surrounding adult brain?

In search for intrinsic factors: does BCL2 regulate the potential of a neuron to extend its axon?

As for extrinsic factors that regulate axonal extension, several molecules and conditions intrinsic to the neuron appear to control axonal growth; such as growth-associated proteins, activated cytoskeletal proteins, Ca^{2+}, cAMP and other second-messenger systems[7,8,27–29]. BCL2 is among the factors that have been discussed in the context of the induction and maintenance axonal growth[23]. The *Bcl2* gene was originally discovered as the proto-oncogene at the breakpoint of the follicular lymphoma translocation t(14;18). It has been shown to be protective against cell death induced by a variety of apoptotic and necrotic stimuli, such as ischemia[30], traumatic brain injury[31], growth-factor deprivation and generation of free radicals[32]. *Bcl2* is expressed in the CNS during the middle to late gestational period and its expression is downregulated at the time of birth, persisting in regions with late differentiation, such as the dentate gyrus[25,33]. The highest levels of BCL2 seen during the embryonic period are found in the neuroblasts of the ventricular zones and in post-mitotic neurons during the period of naturally occurring cell death (NOCD). With aging, BCL2 levels decline in most CNS neurons, but remain elevated in neurons of the PNS. *Bcl2* expression is high in the cortical plate: most prominently around E16. Residual *Bcl2* expression in the adult brain occurs mainly in microglia[25]. However, this interpretation was challenged by a recent study in squirrel monkeys that described two patterns of neuronal *Bcl2* expression[33]: (1) a uniform immunostaining of the entire cell including its processes; and (2) a granular immunostaining that excluded the processes. Over time the first pattern is gradually replaced by the second pattern, which persists in the adult brain. The authors suggest that the former pattern of BCL2 immunostaining is seen in neurons undergoing developmental differentiation while the latter pattern might represent immature undifferentiated neurons that persist in the adult brain[33].

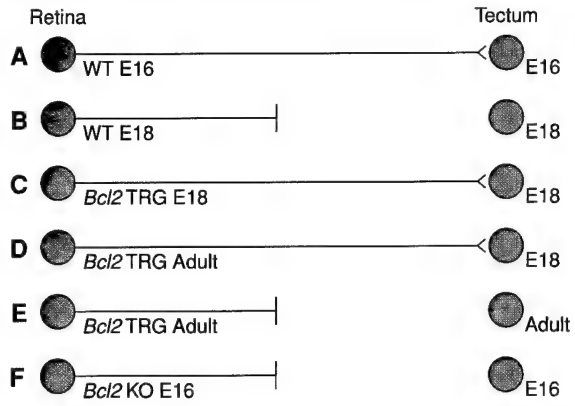

Fig. 2. ***Schematic representation of co-cultures of retina and tectum at various developmental timepoints.*** *Wild-type control embryonic-day 16 (WT E16) retinal ganglion cells (RGCs) show extensive innervation of the tectal target* **(A)**. *At E18 (WT E18), there is a complete failure to innervate the tectum* **(B)**. *Conversely, the RGCs of E18 mice that overexpress* Bcl2 *(*Bcl2 *TRG E18) still show extensive target innervation of the tectum* **(C)**, *as do the same RGCs in adults (*Bcl2 *TRG Adult)* **(D)**. *These RGCs also fail to innervate adult tectum* **(E)**. *RGCs from* Bcl2 *knockout mice (*Bcl2 *KO E16) have lost their ability to innervate tectal tissue already by E16* **(F)**. Data for the figure taken from Chen *et al.*[3]

Naturally occurring cell death is a prominent feature of the developing CNS. It can involve as many as 80% of the neurons, and one of the major functions of histogenetic cell death (a form of NOCD) in the nervous system seems to be the efficient innervation by cells, as a result of competition for growth factors, at the time the cells reach their target[34]. The period of *Bcl2* expression in the developing nervous system coincides closely with the period of NOCD at the time when the axons reach their target[30] and could, thus, be involved in selective sparing of neurons during this period. Mice that overexpress *Bcl2* show reduced neuronal loss during NOCD, which leads to hypertrophy of the nervous system[30]. Interestingly, high levels of *Bcl2* expression also correspond to the entire phase of axonal elongation[25]. Evidence now exists that BCL2 is indeed involved in the process of axonal extension, which is a function that is distinct from its anti-apoptotic activity. In a recent experiment, Chen *et al.*[3] used mice that overexpressed human *BCL2*, coupled to a neuron-specific-enolase promoter. Retino–tectal co-cultures prepared from these mice showed continuous innervation of the tectum at E18, in contrast to wild-type retinal controls (Fig. 2). Incubation with ZVAD-FMK, which is a wide-spectrum caspase inhibitor of apoptosis, prevented cell death as did overexpression of *Bcl2*, but no significant increase in axonal outgrowth was observed[23]. This indicates that effects on axonal outgrowth produced by BCL2 are distinct from its anti-apoptotic effect. Moreover, reduced levels of BCL2 decrease the axonal growth-promoting ability of sensory neurons[35] and in retino–tectal co-cultures prepared from *Bcl2* knockouts at embryonic day 16 (E16), homozygote cultures showed an 80% decrease in innervation of the tectum compared to wild-type controls. This corresponds to the effects observed in wild-type retina co-cultured with tectum at E18, the time when axonal extension has come to an end. Immunohistochemistry of embryonic retinal tissue showed that there was a total loss of BCL2 immunoreactivity in retinas from E18 wild-type mice[3]. This demonstrates that BCL2 levels are directly correlated to the ability of the retinal neuron to extend axons. Axotomized retinal axons in a P4 mouse that overexpressed *Bcl2* grew in large numbers across the lesion site and innervated the tectum at a site that was caudal to the injury[3] (see Fig. 1A,D). Using a dopaminergic cell line, Oh and colleagues[36] showed that vector induction of human *BCL2* leads to robust neurite formations when compared with a control vector. Increased time in culture (more than four days) led to increased neurite length. Zhang and colleagues[37] used a human neural-crest-derived cell line that undergoes spontaneous differentiation and normally expresses moderate levels of BCL2. They showed that manipulations of BCL2 levels with antisense cDNA resulted in a lack of differentiation and that overexpression of *Bcl2* produced by adding sense cDNA resulted in increased axonal outgrowth[37]. The ability of the PNS to regenerate axons and the persistence of BCL2 levels in these cells also suggest that BCL2 has a growth-promoting effect. Notably, *Bcl2* expression has been described in the superior cervical ganglion and the DRG in mice at five months[25].

Concluding remarks

Several extracellular and intracellular factors have been discussed that influence axonal growth in the context of neuron–target interactions *in vitro*, after *in vivo* CNS lesions and by fetal neuronal transplants placed in the adult brain. It can be concluded that, even in the adult brain, it is possible to induce the production of factors intrinsic to the neuron that increase and maintain axonal growth. Further studies of BCL2 and other similar molecules that influence the growth capacity of axons in the adult CNS should, therefore, be instructive.

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REVIEW

Apoptosis in Neuronal Development and Transplantation: Role of Caspases and Trophic Factors

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Fetal ventral mesencephalic (VM) transplants have been studied in the context of dopaminergic (DA) replacement therapy for Parkinson's disease (PD). DA neurons from VM transplants will grow axons and form functional synapses in the adult host central nervous system (CNS). Recently, studies have demonstrated that most of the transplanted DA neurons die in grafts within the first week after implantation. An important feature of neural development, also in transplanted developing fetal neural tissue, is cell death. However, while about 50% of cells born in the CNS will die naturally, up to 99% of fetal cells die after neural transplantation. It has been shown that VM grafts contain many apoptotic cells even at 14 days after transplantation. The interleukin-1β converting enzyme (ICE) cysteine protease and 11 other ICE-like-related proteases have been identified, now named caspases. Activation of caspases is one of the final steps before a neuron is committed to die by apoptosis. Here we review this cell death process in detail: Since the growth of fetal neural grafts placed in the adult brain in many ways mimics normal development, it is likely that the caspases also play a functional role in transplants. Pharmacological inhibitors of caspases and genetically modified mice are now available for the study of neuronal death in fetal neuronal transplants. Understanding cell death mechanisms involved in acute cellular injury, necrosis, and programmed cell death (PCD) is useful in improving future neuronal transplantation methodology, as well as in neuroprotection, for patients with neurodegenerative diseases. © 1999 Academic Press

Transplanting fetal VM neurons has proved that structural brain repair is possible for patients with PD. However, while necessary functional and structural repair is possible using fetal neurons (61, 66), current transplant preparation and procedures are associated with a low yield of surviving DA neurons after transplantation and this limits the potential utility of this treatment (65). During brain development, cell death is believed to occur as a result of the adjustment of neuron number to their trophic factor producing targets (105). Depending on the cellular system, in the range of 20–80% (9, 11, 13, 14, 81, 105) of born neurons die during normal CNS development. However, up to 99% (9, 11, 13, 14) of the transplanted neurons die during graft development. Recent discoveries have provided insight into at least two different kinds of cell death seen in fetal graft development: apoptosis and necrosis (1, 23, 43, 86, 145). A large proportion of cell death in transplants appears to be apoptotic (86, 145). Although many studies show behavioral recovery in rodent models of PD with only 1–4% (97, 98, 112) of transplanted dopaminergic neurons surviving, preventing the massive cell loss seen in neural transplants could improve functional effects as well as reduce inflammation and the presence of immunological stimuli that could lead to transplant rejection. The major current obstacle to transplanting a large number of PD patients with VM grafts is insufficient access to VM DA neurons for grafting. By increasing cell survival in a graft, less tissue would need to be transplanted for functional effects to occur.

Apoptosis, Programmed Cell Death, and Necrosis

Apoptosis or programmed cell death (PCD) is a fundamental biological process in eukaryotes in which individual cells die by activating their own genetically programmed cell death mechanisms (70). The term apoptosis (etymologically from Greek apo-from, detached, separate, and ptosis-falling) was introduced after the discovery that a similar cell death cascade as that seen during PCD also occurs when mature cells are dying as a consequence of some pathological circumstances (70, 135). In the developing nervous system, a large number of cells die before birth by PCD (81, 105).

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PCD is therefore believed to be molding the nervous system's cellular structure and function. The surviving cells retain their ability to undergo apoptosis in adulthood and can display this process during aging and in neurodegenerative diseases (34, 95), such as amyotrophic lateral sclerosis (ALS) (69, 128, 129), Parkinson's disease (94), Huntington's disease (108), Alzheimer's disease (28, 96), and spinal muscular atrophy (68, 126). Neuronal apoptosis is also seen to some degree after acute injuries: trauma (21), ischemia (53), and stroke (67, 79).

There is evidence that both apoptosis and necrosis can occur after similar tissue injury (109, 121). Apoptotic death is distinguished from necrosis by a number of morphological and biochemical criteria (Table 1) (70, 135). The main criterion for apoptosis is the presence of internucleosomal DNA cleavage, seen as a "DNA ladder" on gel electrophoresis, although this is considered a relatively late event (25, 127, 134). However, cases have been described in which apoptosis occurs without demonstrable DNA laddering (25, 26, 103, 127), as in tissues containing diverse cell types where only a subset of cells die by apoptosis. This may be relevant to grafted VM, where *in situ* labeling techniques could be preferable to DNA gel electrophoresis. For VM transplants, double labeling for apoptotic nuclei and dopamine neurons would define the PCD of the therapeutically relevant cell type. Such methodology has been applied in the study of apoptotic dopamine neurons in culture (22, 93, 144). Apoptotic dopamine neurons *in vivo* were clearly described by Macaya *et al.* (85) in a developmental striatal target loss in rodents. *In situ* techniques have proven reliable in studies of susceptibility of developing dopaminergic neurons to undergo apoptosis in response to both 6-OHDA and hypoxic–ischemic injury (88, 104). However, DNA fragmentation may not be an absolute criterion for apoptosis, since certain neuronal populations as exemplified by rat cerebellar granule neurons (118) appear to be able to withstand such a process or show stereotypic biochemical and morphological changes of apoptosis, without clear evidence of internucleosomal DNA cleavage.

Apoptosis can be divided into four phases (Tables 2A and 2B): First, activation of the cell death program is induced by several apoptotic triggers such as deprivation of trophic support. Second, this path toward cell death coincides with metabolic changes such as decreased glucose uptake and reduced protein and RNA synthesis. Beyond a certain point, these changes appear irreversible and the cell reaches the point of "no return," characterized by stereotypic morphological changes in cell structure. Finally, the execution phase is characterized by lysis of the cell (31, 32, 70, 135). The distinction between necrosis and apoptosis is crucial

TABLE 1

Morphological Differences between Apoptosis and Necrosis

Apoptosis (70, 135)	Necrosis (70, 135)
Preservation of membrane integrity	Loss of membrane integrity
Cytoplasmic and nuclear fragmentation (internucleosomal cleavage of DNA/"DNA ladder"), condensed chromatin remains in nucleus	Nuclear flocculation
	Loss of lysosomal contents, loss of cellular content
	Cellular swelling
	Morphological signs of organellar damage
Diminution of cellular volume	Cell lysis, inflammatory response
Plasma membrane blebbing/contortion	
Morphological conservation of organellar structure, retained lysosomal contents	
Apoptotic bodies (budding off of cellular fragments) recognition and phagocytosis, no inflammatory response	

TABLE 2

A. NGF Deprivation: Different Phases of PCD and Apoptotic Events in Dying Neurons (36, 71, 76)

Healthy neuron → Degraded neuron			
1. Activation	2. Propagation	3. Commitment	4. Execution
Apoptotic trigger-like NGF ↓	ROS ↑ Gene expression Glucose uptake ↓ Purine efflux ↑ RNA and protein synthesis ↓	Caspases	Substrate cleavage-like, PARP, and lamins DNA degradation

B. Time Course of Events in Sympathetic Neurons Deprived of NGF (32)

Hours after NGF deprivation	Apoptotic event after NGF deprivation
Propagation phase	
3–8	ROS activation
5–10 (maximum 12–18)	c-jun, c-myb, mkp-1, and cyclin D1 expression
6	Decreased glucose uptake
10	Increased purine efflux
10 (maximum 15–20)	c-fos, fos-b, and NGFI-A expression
12	Decreased RNA and protein synthesis
12–18	No morphological changes
17–20	DNA fragmentation
Commitment phase	
22	"Commitment point"; depending on neuron's age; the older, the more resistent
18–24	Shrinkage and condensation of nuclei
24–30	Significant irregularities
24–36	Atrophied neurons
48	95% of neurons dead with degeneration of cellular organelles

because the latter is under active cell control (39). PCD was first studied in the nematode *Caenorhabditis elegans* (58, 123). Pro- and antiapoptotic genes were found, for which there are mammalian homologues (Table 3), indicating that apoptosis is evolutionary and genetically conserved (141). One such gene is ced-3 which encodes a member of the ICE cysteine protease family and is crucial for the execution of all PCDs in mammals (141). ICE, the first of 12 members of a larger proteolytic family, was initially identified as the protease responsible for proteolytic activation of the interleukin-1β (Il-1β) precursor (24, 100, 115, 130–132). This family of molecules are now known as caspases, standing for cysteine proteases ("c") with cleavage sites after aspartatic acid residues ("aspase"). Caspases are synthesized as inactive proenzymes in the cytoplasm and are activated by cleavage at internally specified conserved aspartate residues, in cells undergoing apoptosis. The caspases thus initiate a cascade of proteolytic cleavage leading to activation of downstream caspases with cellular substrates, such as poly(ADP-ribose) polymerase (PARP) and lamins. Cellular apoptotic mechanisms can involve the Bcl2 family members, mitochondrial release of cytochrome *c,* and the activation of caspases (see Fig. 2): A biochemical sequence for such a process would be the release of cytochrome *c* after opening of mitochondrial permeability pores, which would then activate pro-caspase-3. It has recently been shown that Bcl2 interferes with the activation of caspase-3 by preventing the release of cytochrome *c* (36, 71, 111, 139, 140). BclXL, an antiapoptotic member of the Bcl2 family, may act by holding the proapoptotic Apaf-1/caspase-3 complex inactively bound to the mitochondrial membrane (18, 45, 57, 111). Bax is a proapoptotic homologue of Bcl2 also bound to the mitochondria, which induces the release of cytochrome *c* by heterodimerizing with BclXL and displaces BclXL from the inactive Apaf-1/caspase-3 complex. This activates caspase-3, which cleaves other caspases in the death cascade (18, 45, 111, 114) (see Fig. 2). Neither caspase inhibitors nor Bcl2 has been shown to prevent Bax-induced cytochrome *c* release. Nonetheless, cells overexpressing both Bcl2 and Bax show no signs of caspase activation and survive even though they have significant amounts of cytochrome *c* in the cytoplasm, indicating that Bcl2 can prevent Bax-induced apoptosis by other mechanisms (114) (see Fig. 2).

TABLE 3

Function of Genes Modulating PCD/Apoptosis in *C. elegans* and Mammalians (39, 58, 136, 141)

Gene	Protein	Function	Mammalian homologue
ced-3	CED-3	Vital for cell death	Caspase-3-like-caspases (19, 141)
ced-4	CED-4	Vital for cell death	Apaf-1 (146)
ced-9	CED-9	Prevents cell death	Bcl2-family (80)

Apoptosis and Its Role in Cell Death of Neuronal Transplants and Fetal Development

It may be more appropriate to use the term PCD rather than apoptosis when describing cell death in developing grafts, since the term PCD was originally introduced to describe cell death that occurs specifically during embryonic, fetal, and early postnatal development and thus is a physiological process (82). PCD timing varies from region to region in the brain and is species-specific, though stereotypical. Most probably, PCD is normally the result of competition for a limited amount of target-derived trophic factors and occurs mainly in neurons with axons that have reached their targets (16, 81, 105). This suggests that to some extent synaptic interactions control the survival of neurons during PCD (16, 81, 105). In the developing CNS the survival of neurons depends on access to appropriate amounts of trophic factors (105). During neurogenesis and maturation one-half or more of the number of neurons born are eliminated by PCD (81, 105). During this process, it is believed that the number of neurons in connecting regions is matched to obtain a well-functioning system (59, 105, 125).

The trophic conditions for transplanted fetal cells are different from normal development. For example, the levels of target-derived trophic factors are reduced in the adult compared to the fetal/neonatal brain. Nonetheless, glioblasts contained in the fetal cell preparations develop and produce a glial environment not unlike that seen in normal development. Although many adverse factors influence cells in the process of fetal neural transplantation, such as trauma, oxidative stress, ischemia (144), and lack of growth factors (105), we believe it is reasonable to assume that the process of PCD would continue in the developing transplanted tissue, as it would during normal development. Likewise, the function of PCD in developing VM transplants would be to eliminate cells that are not able to integrate in the developing nervous system, because these cells are possibly unhealthy or maladapted.

The extent of PCD in transplanted fetal substantia nigra is probably more severe than during ordinary development, since there will be greater competition for fewer neurotrophic factors. Moreover, not all of the cells in the fetal VM are dopaminergic and therefore may not respond to the same trophic factors or those available in atypical or ectopic targets (17, 64). The time course of neuronal cell death in nigral transplants has been studied by Barker *et al.* (5). They showed that the majority of DA neurons die during the first 7 days after transplantation. The survival rate of DA neurons is higher in three-dimensional *in vitro* cultures (with the

same dimensions as an *in vivo* graft made out of identical cell suspensions), indicating that features in the tissue environment surrounding a graft are unfavorable to the DA neurons.

Interestingly, Zawada *et al.* (145) showed that growth factors (GDNF, IGF1, and FGF2) reduce apoptosis in VM grafts and thus increase dopaminergic neuron survival. The growth factors appear most effective the first 24 h after grafting, since cell death after the first week was proportional to the number of cells surviving at the first day; 49% versus 32% at 24 h and 26% versus 16% dopaminergic neuron survival at 7 days after grafting in trophic factor-treated and control animals, respectively (see Fig. 1). The number of apoptotic cells was five times greater in 1-day-old grafts compared to 7-day-old grafts in both groups, as assessed by TUNEL technique, indicating that most apoptosis occurs shortly after transplantation. Since only 0.5–10% of the originally transplanted neurons survive after approximately 3 months (9, 12–14) and 16% of dopaminergic cells survive after 1 week, it seems that most cell death takes place in the first week after transplantation (see Fig. 1). Mahalik *et al.* observed many apoptotic cells in 2-week-old fetal mesencephalic grafts and very few apoptotic cells in 4-week-old grafts (86). However, many questions remain. For example, will the rescue of dopaminergic neurons from apoptotic death by trophic factors in the first week of graft development (145) endure? Is apoptosis in the first 2 weeks of graft development fully responsible for the final number of surviving neurons?

Most likely, neuronal death in fetal VM transplants can occur by both necrosis and apoptosis since both mechanisms were observed in developing grafts (1, 23, 43, 86, 145). Previously it was believed that the major mechanism of cell death in developing grafts was necrosis (1, 23). Although neural transplants may exhibit characteristics of both necrosis and apoptosis, one process may dominate (1, 23, 86, 145). Recent studies have demonstrated a continuum between apoptosis and necrosis (36, 109, 121). They show that the same type of stimulus can lead to either apoptosis or necrosis, depending on the concentration of the toxic agent, such as glutamate receptor agonists (35, 109). Low concentrations induce apoptosis; high concentrations induce necrosis. Of note, Bcl-2 is able to block both apoptosis and necrosis, suggesting that some events underlying apoptosis and necrosis may be similar (119).

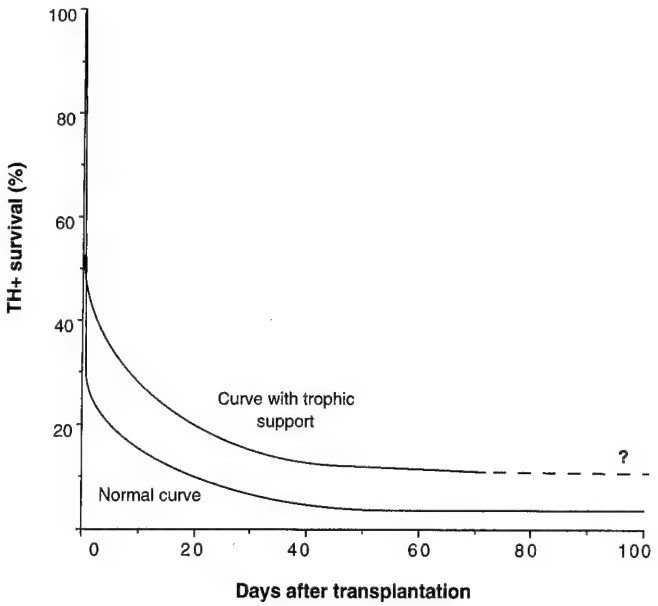

FIG. 1. An estimated *in vivo* dopaminergic neuronal survival rate in ventral mesencephalic (VM) grafts placed in the adult rat brain from several independent research groups. Notably, there is considerable (up to 99%) cell death associated with the transplantation paradigms. Since approximately 50% of neurons die during normal development, it seems reasonable that optimization of neuronal survival in fetal transplants is possible. Based on data from Zawada *et al.* (145), major cell loss occurs during the first days after transplantation (26% survival after day 1). At 7 days, this value had decreased to 16% in that study. Survival rates at 4–5 weeks vary considerably (1.5 to 7%) (98, 112). At longer survival times, the percentage survival ranges between circa 0.5 and 10% (8, 9, 13, 14). In similar fetal ventral mesencephalic cell preparations treated with trophic factors (GDNF, FGF2), the survival rates are higher. Zawada *et al.* (145) found a 49% survival of TH+ neurons 1 day after transplantation with a combination treatment of GDNF, IGF1, and FGF2. These grafts displayed a 32% survival at 7 days. Estimates from studies at 4 weeks by Rosenblad *et al.* (112) indicated a 2-fold increase in DA survival of cell suspension grafts in the presence of GDNF (although the total percentage survival was lower than in previously cited studies). In VM solid tissue grafts, Granholm *et al.* (47) also found a 2.6-fold increase in cell survival at 6 weeks after transplantation with GDNF treatment compared to untreated tissue, which would suggest an 18% survival of TH+ neurons based on the estimated 7% survival rate of TH+ neurons in similar untreated solid VM tissue grafts after 10 weeks according to Haque *et al.* (52).

STRATEGIES TO DECREASE CELL DEATH IN FETAL NEURONAL TRANSPLANTS

Cell Preparation Techniques in Transplantation Paradigms

The first fetal nigral grafts were implanted as solid pieces into adult rats with DA loss of the striatum (10) or as tissue pieces in the ventricle adjacent to the caudate nucleus (107). Subsequently, methodology for transplantation of dissociated cell suspensions of fetal VM was developed, providing many alternative techniques for preparing the tissue prior to transplantation (37). Many basic variables such as gestational age, dissection procedures, and dissociation media can influence the viability of the grafted neurons. For example, variations in proteolytic enzymes such as trypsin, use of DNase, and amount of trituration can influence the outcome. Barker *et al.* (6) demonstrated that optimal neuron survival scores were obtained in VM tissue from rat embryonic day 13 and 14 (E13–E14) fetuses when

incubated in purified 0.1% trypsin solution for 60 min and triturated using a fire-polished Pasteur pipette. We estimate that the survival rate of tyrosine hydroxylase positive (TH+) neurons in their studies ranged from approximately 2.3 to 18% in 4-month-old grafts depending on which brands, concentrations, and incubation times of trypsin were used (6). As far as surgical procedures influence outcome, the micrografting technique is one of the most recent modifications of experimental CNS transplantation techniques. This technique allows precise and reproducible injection of VM cell suspensions at single or multiple sites with minimal trauma, which yields improved graft survival and integration of the grafted neurons (101, 102).

Trophic Factors

Since with current neurotransplantation procedures only 0.5–10% of transplanted fetal neurons survive (9, 12–14), it is of interest to enhance this fraction of living cells. For practical purposes, current transplant protocols for PD patients require as many as 10–15 fetuses to obtain a set of transplantable VMs to provide a sufficient number of DA cells to help the patient (9, 13). Neurotrophic factors are able to promote the survival of DA neurons (see Fig. 2). For example, pretreating VM neurons with basic fibroblast growth factor (FGF2) followed by repeated FGF2 intrastriatal injections increases the survival of TH+ neurons 100% (90). In addition, cografting of FGF2-transfected fibroblasts together with fetal dopamine cells causes augmented survival and fiber outgrowth of transplanted DA cells (124). Infusions of brain-derived neurotrophic factor (BDNF) in VM grafts produce enhanced striatal DA innervation of the host (143). Intrastriatal infusions of glial cell line-derived neurotrophic factor (GDNF) dose-dependently enhance DA cell survival and fiber outgrowth from VM grafts (120). Granholm *et al.* (47) showed increased size of transplanted VM tissue pieces, dopaminergic cells, and fiber outgrowth after pretreating VM tissue GDNF. Furthermore, Rosenblad *et al.* (112) showed that infusions of GDNF adjacent to intrastriatal VM grafts improve TH+ neuron survival and TH+ fiber outgrowth. Sautter *et al.* (116) showed that genetically modified GDNF-releasing capsules implanted near intrastriatal dopaminergic cell grafts show increased DA neuron survival and fiber growth toward the trophic factor-releasing capsule. As mentioned previously, Zawada *et al.* (145) demonstrated that GDNF, IGF1, and FGF2 prevent apoptosis in mesencephalic grafts. The most substantial effects are seen in grafts that are treated continuously with trophic factors after transplantation (20, 30, 90, 113, 116, 124, 143). Trophic factors cannot be given systematically because of the near impermeable blood-brain barrier (BBB). Infusions of trophic factors can, however, be made directly into the CNS area of transplantation (90, 143). To overcome the BBB, trophic factors can be conjugated with antibodies against the transferrin receptor, cross the BBB, and result in increased graft survival (2, 3, 41, 46). Such transferrin receptor antibody-based delivery systems may also be useful in transporting other large molecules, such as pharmacological caspase inhibitors.

Another way of providing trophic factors to fetal neural grafts is by cotransplantation of genetically modified cells to produce increased graft survival (20, 30, 116, 124). Analogous to trophic factor enhancement is cografting of fetal cells with their fetal target tissue. In cografting of VM and striatum (LGE), a denser innervation area in the striatum of the host brain can be seen compared to VM cells alone (15). Yurek *et al.* also found larger TH+ cell bodies and more fibers in the VM/LGE cografts implanted into the striatum of rats with unilateral 6-OHDA lesions (142). Such cotransplants increased function, cell survival, and reinnervation of the lesioned adult striatum (27). BDNF-producing E18 striatal astrocytes cotransplanted with fetal VM tissue also promoted the survival of dopaminergic neurons in the denervated striatum. However, postnatal day 0 (P0) striatal BDNF-secreting astrocytes transplanted together with fetal nigral cells impaired graft survival. BDNF was not able to overcome the influence of the astrocytes' age on cell survival (74). As a corollary, cotransplantation of human NGF-producing fibroblasts and adrenal chromaffin cells in the striatum of hemiparkinsonian rats increased long-term functional survival (29).

Overall, the observations that a significant proportion of transplanted neurons die by PCD are consistent with the neurotrophic theory, which states that developing neurons are initially overproduced and then compete for limited amounts of target-derived survival-promoting factors as to prevent their death (17, 63, 64, 81, 105).

Lazaroids, Antioxidants, cAMP, Ascorbic Acid, and Calcium Blockers

Lazaroids are 21-amino steroids with antioxidant activity, which inhibit free radical generation and lipid peroxidation. Treating VM cell cultures with lazaroids U-74389G and U-83836E enhanced the survival of TH+ neurons (44). Moreover, identical lazaroids prolonged cell viability in dissociated fetal VM cell suspensions prior to grafting and increased the survival of dopaminergic neurons 2.6-fold following transplantation (97). Utilizing lazaroid U-83836E in medium at 4°C for 8 days in fetal VM tissue before culturing and striatal transplantation enhances the survival of dopaminergic neurons compared to untreated control VM tissue (50). U-83836E, used at a very low concentration, protected against oxidative stress in cultured cortical neurons exposed to beta-amyloid toxicity (84). In addi-

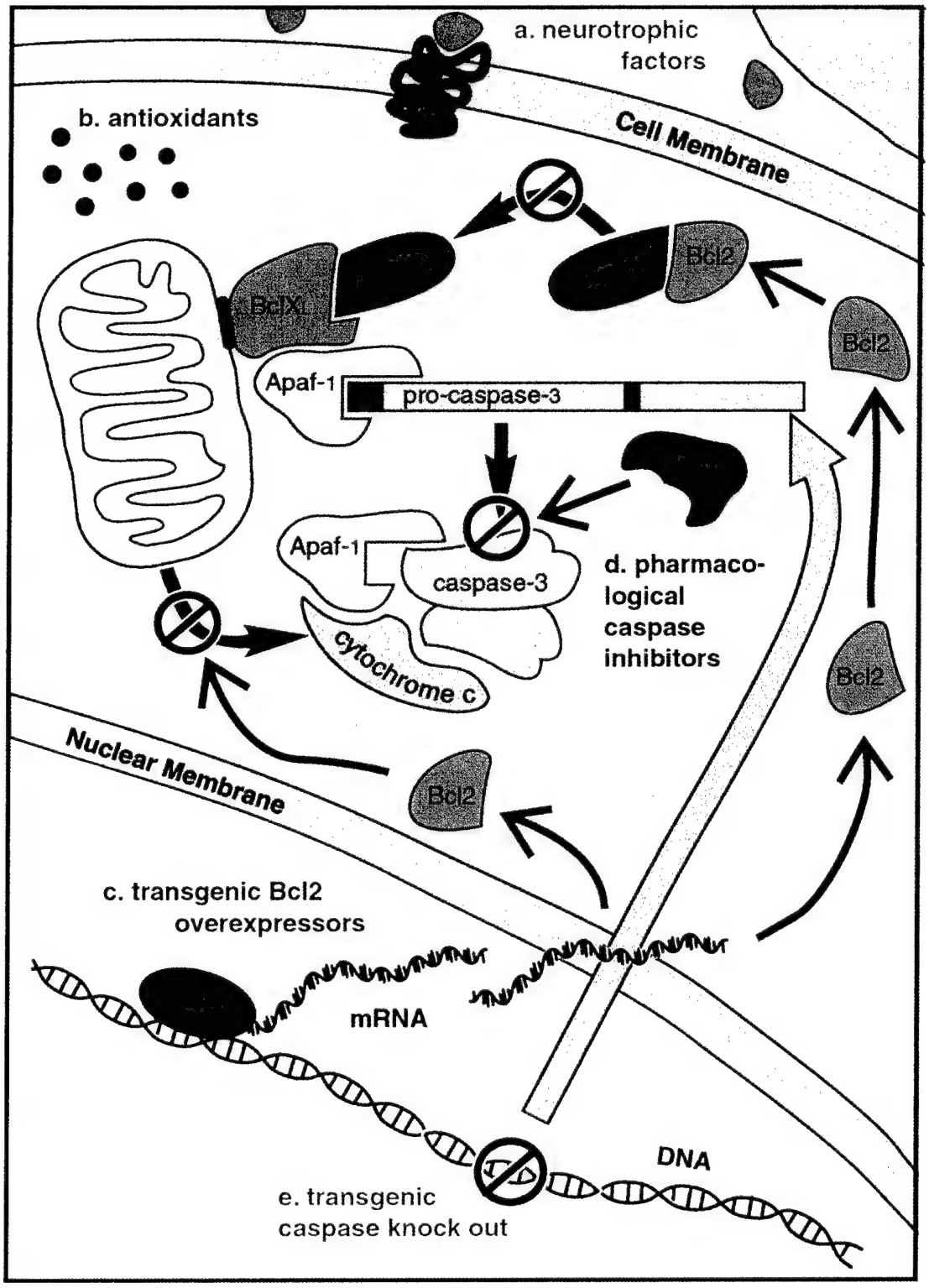

FIG. 2. Possible mechanisms that increase TH+ neuron survival in fetal mesencephalic grafts. (a) Grafts pretreated with trophic factors before transplantation and/or after grafting show increased dopaminergic neuron survival. Cografting of neurons with their target increases graft survival presumably by target-derived trophic factors. (b) Treatment with lazaroid and other antioxidants increased neuron survival in grafts. (c) Transplantation of neuronal grafts overexpressing Bcl2 may increase survival since Bcl2 plays an important role in preventing apoptosis. The Bcl2 family has a double role in preventing activation of caspase-3, one of the most effective executioners of apoptosis. BclXL keeps the apoptotic Apaf-1 (apoptosis protease activating factor)/pro-caspase-3 complex inactively bound to the mitochondrion and Bcl2 prevents permeability from cytochrome *c* (presumed, Apaf-2) of the mitochondrion so that its binding to Apaf-1/pro-caspase-3 is prevented and

tion, U-83836E is neuroprotective to glutathione-depleted embryonic mesencephalic neurons (48) and prevented their death induced by congeners of nitrogen monoxide (49). Tirilazad mesylate, the single lazaroid approved for human use, has been found to improve the survival of both rat and human fetal VM cells *in vitro* (106).

In experiments in which neurons were taken from either transgenic mice overexpressing superoxide dismutase (SOD) (98) or adenovirus modified to overexpress SOD (4), similar survival effects to those observed in the lazaroid experiments were observed. Grafting of tissue derived from the SOD transgenics resulted in improved cell viability prior to grafting and a fourfold increase in TH+ neuronal survival following transplantation (98). However, the adenovirus-modified SOD-expressing grafts failed to show significant increase in dopaminergic neuron survival, although a trend was seen (4).

Dibutyryl cyclic AMP (dbcAMP), a cell membrane-permeable analogue of cyclic AMP (cAMP), attenuates apoptotic death and promotes maturation of cultured TH+ mesencephalic dopaminergic neurons (54, 93). dbcAMP was most effective when added immediately after cell plating, but delayed treatment also prevented degenerative processes (93). These results suggest that additional factors can be tailored for neuroprotective effects; for example, the survival-promoting action of GDNF on DA neurons appears to require activation of cAMP-dependent signaling pathways (40).

Pharmacological Inhibition of Caspases

A powerful strategy to decrease cell death in dissociated cell suspension or dissected tissue pieces would be treatment with caspase inhibitors prior to transplanting these fetal cells into the host brain (see Fig. 2). There are several potent caspase inhibitors available and we will first describe a few current examples of how they have been applied to neural cells in preventing apoptosis. There are several examples of induction of caspase(s) during apoptosis in neurons *in vitro* and *in vivo*.

Intracerebroventricular (icv) administration of an irreversible ICE/caspase-1 inhibitor, z-VAD-DCB, markedly reduces brain damage after focal cerebral ischemia in the rat (83). Yakovlev *et al.* found that caspase-3-like proteases are activated after traumatic brain injury in rats and demonstrated that blockade of these caspases by inducing z-DEVD-fmk (a relatively specific inhibitor of caspase-3) icv before and after injury markedly reduced posttraumatic apoptosis as shown by DNA electrophoresis, TUNEL staining, and improved neurological recovery (137). Haviv *et al.* (55) studied apoptosis in neuronal PC12 cells deprived of trophic factor and found that caspase-3 or caspase-3-like proteases, but not caspase-1, are induced during the process. Both the viral caspase inhibitor gene p35 and broad spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) inhibit the death of neuronal PC12 cells (55). Definitive proof of caspase-3 involvement was obtained by Northern and Western blotting, demonstrating that PC12 cells express caspase-3 and that cleavage of caspase-3 substrates occurs in cell lysates prepared from trophic factor-deprived PC12 cells. Consistent with these findings, a previous study had shown that the viral apoptosis suppressor gene p35 was neuroprotective in an immortalized mesencephalic cell line (110). Moreover, caspase-1-specific inhibitors, namely Ac-Try-Val-Ala-Asp-chloromethylketone (Y-VAD.CMK), Ac-Try-Val-Ala-Asp-aldehyde, and crmA, a cytokine response modifier gene and a viral caspase inhibitor, were much less potent in inhibiting cell death (55). Of note, Y-VAD.CMK is relatively specific for caspase-1, but to a certain extent also blocks caspase-4 (87).

In the context of oxidative stress, Du *et al.* (36) found that exposure of cerebellar granule cells to low concentrations of MPP+ results in apoptosis and that the specific caspase-3 inhibitor acetyl-DEVD-CHO markedly attenuates such apoptotic death. Interestingly, necrosis of sucrose-cultured granule neurons occurred when the level of oxidative stress was increased by elevation of MPP+ concentrations (36). Moreover, cytoplasmic extracts from low-dose MPP+-treated cells contained protease activity that cleaved acetyl-DEVD-*p*-nitroaniline, a caspase-3 substrate (36). The more specific inhibitor of caspase-1, acetyl-YVAD-CHO, was ineffective against low-dose MPP+ neurotoxicity (36). Notably, cytochrome *c*, also known as Apaf-2 [one of the three apoptosis protease-activating factors (57)], was increased just before onset of apoptosis (36).

NGF-deprived sympathetic rat neurons in culture show reduced apoptosis when treated with Bocaspartyl(OMe)-fluoromethylketone (BAF), an inhibitor of both caspases-1 and -3 (33). BAF did not prevent decreased protein synthesis or increased expression of c-jun, c-fos,

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activation of caspase-3 will not occur. The following may happen when a death stimulus activates the apoptotic pathway. Bax will displace BclXL from Apaf-1/pro-caspase-3 and cytochrome *c* will bind to Apaf-1/pro-caspase-3. This will result in activation of pro-caspase-3 and activation of the caspase cascade will lead to cleavage of critical cellular substrates resulting in dissolution of the cell. The Bcl2 family is able to inhibit this process. (d) Pharmacological caspase inhibitors obstruct caspase activation and therefore cell death. Pretreating fetal mesencephalic grafts with caspase inhibitors may increase dopaminergic neuron survival. (e) Transplanting neural tissue from transgenic knock out (ko) mice may also increase neuron survival.

and other mRNAs that occur in the process of neuronal apoptosis (Tables 2A and 2B). This indicates that the caspases function downstream of several transcriptional events during apoptosis (33). Addition of either NGF or BAF prevented cell death of NGF-deprived sympathetic neurons at similar time points (33).

Thus, there are a number of factors to be determined for obtaining effective pharmacological caspase inhibition for fetal neural cell transplantation. Generally, it is useful to define which specific caspase(s) is involved in cell death of the specific cell type transplanted (54, 91, 93).

Inhibition of Caspases: Gene Deletion in Mice

An alternative to the pharmacological strategy of caspase inhibition is to transplant neural fetal tissue from mice lacking genes encoding caspases (see Fig. 2). Many different mice with a targeted endogenous gene disruption for one of the caspases have been developed over the past few years. For example, ICE/caspase-1-deficient mice (76) (Table 4) show no phenotypic abnormalities in the brain (76). Mice that express a dominant negative mutant of caspase-1 (42) and caspase-1-deficient mice (117) are protected against ischemia-induced brain damage. Thus, in some cells caspase-1 depression helps prevent apoptosis, while its role in PCD in brain development does not appear significant (76). CPP32-, Yama-, and apopain/caspase-3-deficient mice (77) (Table 4) show deviant brain development as a consequence of decreased developmental apoptosis, indicating that caspase-3 plays an essential role during morphogenetic cell death in the mammalian brain. In this study, the entire brain was larger, and in such caspase-3-deficient mice, there were significant hyperplasias, ectopic cell masses (in cortex, cerebellum, striatum, and hippocampus), double brain structures (in cortex and optic stalk), and disordered cell layering. No apoptotic cells were found along ventricular walls, optic stalk, or spinal cord, locations ordinarily packed with apoptotic cells during CNS neurogenesis. Caspase-3-deficient mice suffer from premature lethality and are not fertile. Surprisingly, caspase-3-deficient thymocytes were evenly sensitive to various apoptotic stimuli compared to the wild-type (wt) thymocytes, indicating that caspase-3 is specifically activated in the brain. Woo *et al.* (133) found evidence that caspase-3 activation is stimulus- and tissue-dependent. Moreover, caspase-3 is constitutively expressed in adult brain and is activated after ischemic attacks (99). However, note that one study found that most types of human brain and spinal cord neurons had little or no caspase-3 expression (73).

ICH-1/caspase-2 is highly expressed during embryonic days 9 and 16 in the brain and various other tissues. ICH-1/caspase-2-deficient mice (7) (Table 4) show no obvious cellular or histological brain abnormalities. However, fewer neurons were found in the facial nuclei in the caspase-2-deficient newborns compared to the wt mice. At embryonic day 16.5 (at a time when PCD still occurs), the facial neuron number was equal in knock-out (ko) and wild-type mice, indicating that accelerated apoptosis had occurred rather than a decreased production of neurons.

Recently, a new caspase ko mouse has been introduced by Kuida *et al.* (76), namely the ICE-LAP6/Mch6 or caspase-9-deficient mouse. The majority of caspase-9 ko mice die perinatally with a marked enlarged and malformed brain, most severe within the cortex and forebrain, as a result of reduced apoptosis during development of the nervous system. No apparent malformations are found in spinal cord and other parts of the fetal body. At different embryonic stages there are abnormalities in the brain, generally a larger proliferative zone devoid of pyknotic clusters and fewer apoptotic cells in both forebrain and midbrain. For example, at E16.5, the ko brain shows an increased number of proliferative cells as well as protrusion of the midbrain from the skull. The ectopic brain masses are enlarged and irregular on the surface. Caspase-9−/− mice are smaller than control littermates, and most die 3 days after birth. Caspase-9 deficiency prevents caspase-3 activation in fetal mice brains. In addition, cytochrome *c*-mediated cleavage of caspase-3 is absent in the cytosol of caspase-9-deficient cells but is restored after addition of caspase-9, suggesting that caspase-9 acts upstream of caspase-3 in certain apoptotic pathways (75). Moreover, these observations may confirm that caspase-9 is APAF-3 in the proapoptotic Apaf-1/cytochrome *c* (APAF-2) multiprotein complex which activates pro-caspase-3 during apoptotic death (see Fig. 2) (78). The caspase-9-deficient thymocytes are resistant to a variety of apoptotic stimuli, for example, anti-CD3 plus anti-CD28, etoposide, γ-irradiation, and dexamethasone (51, 75), but are equally susceptible to apoptosis mediated by Fas (75), UV irradiation, and anti-CD95 (51) compared with wt thymocytes. The caspase-9-deficient thymocytes' resistance to some apoptotic stimuli is consistent with the delayed DNA fragmentation and absence of caspase-3-like cleavage (75). Caspase-9-deficient embryonic stem cells and embryonic fibroblasts are also resistant to UV light and γ-irradiation, although cytochrome *c* is translocated in the cytosol of caspase-9-deficient embryonic stem cells after UV stimulation, suggesting that caspase-9 acts downstream of cytochrome *c* (51). Moreover, caspase processing is halted in embryonic stem cells but not in thymocytes or splenocytes. These studies show that the requirement for (caspase-3 and) caspase-9 in apoptosis is cell type- and stimulus-specific, indicating the existence of multiple apoptotic pathways (Table 4) (51).

Thus, reducing apoptosis in neural transplantation may be possible by using fetal dopaminergic cells from

TABLE 4

Different Caspase-Deficient Mice, Their Morphology, Function, and Protection against Induced Apoptosis and Trauma

Mouse deficient for	Brain morphology	Body morphology	Altered functions	Induced apoptosis/ trauma
Caspase-1 (87)	Normal	Normal	IL-1β, Il-1α, TNF-α, and IL-6 production after LPS induction	Monocytes (87) and mice (90) resistant to endotoxic agent LPS Thymocytes sensitive to dexamethasone and ionizing radiation, resistant to anti-Fas antibody (87) Decreased brain damage and faster functional recovery in ischemic models (51, 65)
Caspase-2 (8)	Normal, except for less motor neurons in facial nucleus at E 16.5	Excessive germ cells in ovaries	Oocyte death	Oocytes resistant to chemotherapeutic drugs B-cells resistant to granzyme B and perforin but sensitive to several agents[a] (8), accelerated cell death in NGF-deprived sympathetic neurons
Caspase-3 (88)	Larger brain with hyperplasias, ectopic cell masses, disorganized cell deployment, double brain structures, and less pyknotic cells	Smaller, die at 1–3 weeks of age	PCD in CNS	Thymocytes are sensitive to the following stimuli[c] (88) Lymphocytes are resistant to anti-CD95 plus anti-CD3, UV, and γ-irradiation (159) Splenocytes are sensitive to UV and γ-irradiation (63) Embryonic fibroblasts are resistant to[b] (159)
Caspase-9 (86)	Enlarged malformed brain-like protrusions of brain mass, stenosis of ventricles, heterotopias, invagination, and interuption of the telencephalic wall with less pyknotic cells	Smaller, most die before postnatal day 3	PCD in CNS Activation of caspase-3	Thymocytes are resistant to the following stimuli: dexamethasone, γ-irradiation, etoposide, and anti-CD3 plus anti-CD28 (63) and are sensitive to α-CD95, TNF-α, UV, shock, sorbitol (63), and Fas (86) Splenocytes are resistant to γ-irradiation and are sensitive to UV, sorbitol, adriamycin, etoposide, and *cis*-platinum (63) Embryonic stem cells are resistant to all stimuli used in splenocytes (63) Embryonic fibroblasts are resistant to[a] (159)
Caspase-11 (158)	Normal	Normal	IL-1β and Il-1α secretion after LPS induction	Mice resistant to LPS Fibroblasts resistant to caspase-1 overexpression

[a] Anti-Fas antibody, doxorubicin, etoposide, γ-irradiation, and stauroporine.
[b] UV, γ-irradiation, adriamycin, and etoposide.
[c] Anti-Fas antibody, dexamethasone, C2-ceramide, stauroporin, and γ-irradiation.

transgenic mice deficient for one particular caspase. There are currently at least five different caspase ko mice, respectively caspases-1, -2, -3, -9, and -11 (Table 4). In caspase-3 ko, the brain is predominantly affected relative to other body regions as a result of failed neural apoptosis during CNS development. Although the brain abnormalities observed in caspase-3 and -9 kos are strikingly similar, it appears that caspase-9 ko brain is more severely affected than caspase-3-deficient brain. The similarities of the phenotypes indicate that caspases-3 and -9 may act along the same biological pathway during brain development. Furthermore, it has been shown that caspase-9 is an upstream activator of caspase-3 (76) and this may be why caspase-9 ko brain is more affected than caspase-3 ko brain. Nonetheless, the other mutant caspase mice (caspases-1, -2, and -11) have normal phenotypes, indicating a redundant role for these caspases during CNS development. How-

ever, it is not known if these neurons are functionally altered. Modifying these caspases may still be protective for neurons in a fetal neural transplant and may therefore serve as alternative donor sources.

The differences in brain development and other characteristics seen between caspase-3 ko and caspase-1, -2, and -11 kos may be due to, first, the fact that caspase-3 and caspases-1, -2, and -11 are functionally different: respectively "effector" caspases (caspases-3, -6, -7, and -9) and "regulator" caspases (caspases-1, -2, -3, -4, -5, -8, and -10). This distinction is based on the different lengths of the caspases' N-terminal prodomains, assuming that this may be of functional importance. Effector caspases have short prodomains while regulator caspases have long prodomains. In a hypothetical cascade, the regulator caspases operate upstream and the effector caspases act downstream to the cascade cleaving crucial substrates (100, 130). *In vitro* studies have demonstrated the existence of such a caspase cascade but to what extent this occurs *in vivo* is still unknown (115, 122). There may also be tissue-specific activation of caspases. Moreover, not all caspases may be involved in developmental PCD, although they could still play a role in nondevelopmental cell death.

There are other genetically modified mice with decreased apoptosis that could be useful as donor tissue for fetal dopaminergic transplants, for example Bcl2 overexpressors (see Fig. 2) and JNK3 (c-Jun amino-terminal kinase) ko mice. Bcl2 overexpressing mice show reduced neuronal loss during PCD, leading to hypertrophy of the CNS. Adult mice are less susceptible to ischemia; they show reduced brain infarction after middle cerebral artery occlusion (89). Additional evidence for Bcl2's regulator role in neuronal development and PCD is that Bcl2-deficient mice showed progressive degeneration of several neurons in the CNS after the physiological PCD period (92). In SOD mice with overexpressed Bcl2 in their genome, onset of ALS-like degeneration was delayed (72). Another enzyme involved in stress-induced cell apoptosis is JNK3. Adult JNK3-deficient mice are healthy and fertile, look normal, and show no apparent abnormalities in different brain regions (138). JNK3−/− mice are protected against glutamate neurotoxicity (138). We are currently investigating survival and axonal growth of fetal VM tissue obtained from Bcl2 overexpressors in animal models of PD (62).

Pharmacological Caspase Inhibitors and/or Caspase Gene-Deficient Tissue Transplantation with Trophic Factors

Pretreating neural grafts with pharmacological caspase inhibitors or transplanting VM tissue deficient for a specific caspase in the deafferented striatum appears to increase the survival of DA neurons in transplants (56). However, it is not yet known if the neurons saved by inhibiting caspases are unhealthy or dysfunctional, since they are prevented from dying in the last stages of the apoptotic pathway. It is questionable whether it is always beneficial to block a cell death program since cells may be already dysfunctional before the caspases are actually activated and execute cells. It might be beneficial to lose these unhealthy neurons in order to obtain a well-functioning integration between the grafted fetal neurons and the host brain. An example that may illustrate this point is the neurodegenerative disease ataxia-telangiectasia (AT), where it appears that the lack of appropriate cell death mechanisms of damaged neurons can lead to accumulation of dysfunctional neurons, which then degenerate at a later stage (60). Thus, it is conceivable that in some situations preventing physiological PCD may lead to unforeseen disturbances in normal graft development and experiments are needed to illuminate these issues. It has been shown that treating fetal mesencephalic transplants with trophic factors also increases survival, fiber outgrowth, and functional recovery in models with PD (see above). Adding NGF to trophic factor-dependent neurons prevents the neurons from dying by blocking a crucial posttranslationally late event in neuronal PCD (32, 33, 38). However, NGF can only abort PCD before the neurons are irreversibly committed to die (31–33). Presumably, the caspases are activated during the commitment phase or just before the execution phase of PCD (Tables 2A and 2B).

In conclusion, to maximize the use of the protective strategies discussed, it may therefore be possible to combine pharmacological caspase inhibitors and trophic factors in neural transplants. The trophic factors can prevent neuronal death by providing a growth stimulatory effect for the neurons, while caspase inhibitors prevent neurons from reaching the irreversible biochemical executive point for a cell death process to occur. This suggests that combinations of antiapoptotic agents and trophic factors might act synergistically in blocking cell death.

The current understanding of neuronal PCD in normal brain development now includes knowledge of intricate intracellular cascade mechanisms, including caspases, that serve in the larger bioadaptive context to control the size of neuronal brain cell populations. This understanding can now be experimentally advanced in neurotherapeutic efforts to treat neurodegenerative disease by neural transplantation.

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CHAPTER 8

Dopamine Neuron Grafts: Development and Molecular Biology

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CHAPTER 8

Dopamine Neuron Grafts: Development and Molecular Biology

Lauren C. Costantini and Ole Isacson

The introduction of grafting dopaminergic (DA) neurons into rodent host brains illustrated the potential of this technique for both experimental and clinical applications.[1,2] Basic research utilizing these transplants has revealed information regarding development and connectivity of CNS neurons, while studies aimed at therapeutic strategies for Parkinson's disease (PD) have shown the potential of this procedure for "biological replacements", reconstructing the circuits within a degenerated brain.[3] The current experiments involving fetal neural grafts provide information about mechanisms and processes involved in phenotypic DA neuron development, and serve as a guide to alternative cell sources for clinical neural transplantation.

Establishment of Surviving Dopamine Neuron Grafts

Studies have demonstrated that embryonic day (E) 14 for rodent tissue[4] and 6.5-9 weeks post-conception for human tissue[5] are the optimal ages of ventral mesencephalic (VM) donor tissue for DA neuronal survival and functional effects when transplanted into the DA-denervated striatum. The minimum number of surviving transplanted DA neurons required for functional effects to be revealed in animal models is approximately 100-200.[6] Since only 10% of the transplanted VM cells are DA, and only 1-10% of these DA neurons survive,[7-9] as many as 10-15 fetal VM per patient may be required for sufficient survival and reinnervation.[10] Strategies to improve the survival of the DA neurons within these grafts are being considered, including treatment with growth factors, antioxidants, cotransplantation, and modified implantation procedures.[11-15]

Enhancing Cell Survival with Growth Factors and Target Tissue

Methods to sustain the development and function of embryonic VM DA cells after transplantation into DA-depleted striatum are currently under investigation. Elements which are crucial for the maturation and connectivity of neurons during normal development of the brain may also play a role in the development and integration of grafted embryonic tissue. Based upon the observations that several neurotrophic factors affect the innervation of targets and the survival of neurons during development, administration of these factors along with transplanted "developing" fetal DA neurons has been examined. Among the trophic factors that can enhance development of DA neurons after grafting into rodent are brain-derived neurotrophic factor,[11,16] basic fibroblast growth factor,[17] and glial cell line-derived neurotrophic factor.[18-20] Technical strategies have included exposure of the fetal cells to neurotrophic factors prior to transplantation[16,17,21] or by administration after transplantation.[11,18-20]

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The target-derived neurotrophic factor hypothesis predicts the presence of DA-trophic activity in striatal tissue, and several studies support this hypothesis. Based on in vitro and in vivo observations of the enhancing effects of striatal tissue on nigral DA cell development and survival,[22-24] we have demonstrated that inclusion of embryonic striatal cells,[14] specifically from the lateral ganglionic eminence, enhanced the survival of DA neurons in VM transplants, and also required the transplantation of fewer nigral cells to produce a marked behavioral effect.[13]

Protecting Transplanted Cells with Antioxidants

The poor survival of DA neurons after transplantation may be caused by damage to the VM tissue during preparation, or via cell death after transplantation such as that seen during development.[25] A large proportion of DA cell death occurs during the first two weeks after transplantation due to these factors, and/or due to suboptimal conditions in the host brain during the early phases after transplantation.[26] One hypothesis for the mechanism of this acute cell death is formation of free radicals during the process of dissociation. This is supported by evidence that VM from transgenic mice overexpressing Cu/Zn superoxide dismutase, the major free radical scavenging enzyme, produces transplants with four-fold greater survival of DA neurons and more extensive functional recovery.[12] Treatment of transplanted cells with antioxidants increased the yield of surviving DA neurons, which correlated with an earlier onset of graft-induced functional effects.[12,27] These findings have recently been taken into the clinical setting: One inhibitor of lipid peroxidation, tirilazad, has been included in the solutions for storage and preparation of donor tissue in the clinic; in addition, the patients are treated with this lazaroid for 72 hours after transplantation.[28]

Utilizing Medial VM for Dopamine Grafts

Since several studies investigating optimal donor age have concluded that E13/14 (rat) yields most successful transplants,[29] other issues seem critical. The selection of the most appropriate region for dissection is therefore of great importance. Presently, the embryonic dissections utilized for transplantation typically contain 2-10% DA neurons while the remainder are of other neuronal phenotypes, such as GABAergic.[7,29] Other neurons within a developing transplant may hinder the survival and growth of the small proportion of DA neurons, since neurons compete for the limited supplies of trophic support for survival during development.[30] Therefore, obtaining an initially enriched suspension of DA neurons may enhance the development of the transplants.

Cells generated from the ventricular zone at E11 migrate ventrally and then move laterally to form the substantia nigra pars compacta (SNc) and ventral tegmental area. Tyrosine hydroxylase (TH; a synthetic enzyme involved in production of DA) immunoreactivity can be detected at E12.5 in the medio-basal region of the mesencephalon. By E14, TH^+ cells are located laterally along the ventral surface to form the primordia of the SN.[31,32] In order to enhance the relative proportion of DA neurons, we compared numbers of DA neurons from tissue dissected from the medial portion of the VM versus the lateral VM (Fig. 8.1). A higher proportion of TH^+ neurons were observed in primary cultures of medial VM when compared with lateral or whole VM.[33] Evaluation of E16 solid VM transplants revealed a larger number of surviving TH^+ cells in grafts from medial region of VM compared to lateral VM.[34]

This higher proportion of DA neurons in medial VM can be attributed to the presence of a higher number of DA neurons in the medial region of the VM at E14. Alternatively, the higher proportion of TH^+ neurons in medial VM may represent enhanced survival of DA neurons in medial VM. Dopamine neurons are responsive to a variety of trophic factors such as GDNF,[35] bFGF,[36] TGF,[37] and BDNF.[38] A more pure population of DA neurons present

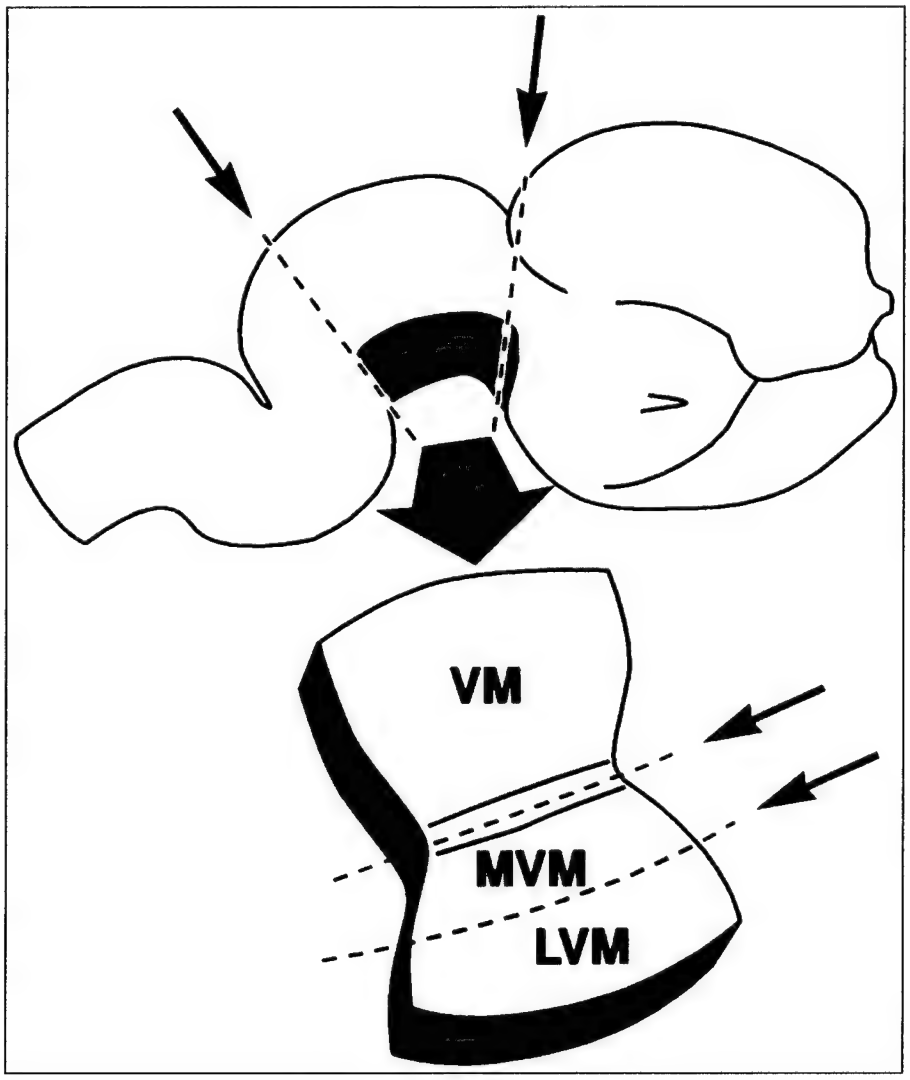

Fig 8.1. Dissection of E14 medial and lateral ventral mesencephalon (VM). Dashed lines through cartoon of entire brain show dissection of VM; dashed lines in enlarged VM region show differential dissection of medial VM (MVM) and lateral VM (LVM). Reprinted with permission from Neuroreport 1997; 8:2253-2257, ©Rapid Sciences.

in medial VM may yield less competition from other cell types for trophic factors, and thus enhance the survival of the relatively small proportion of DA neurons present, since receptors for some growth factors have been detected throughout the SN.[39] If this is the case, the development of DA neurons after transplantation would be hindered by the presence of other cells. This hypothesis is consistent with findings in nigro-striatal cotransplantation studies: When equal numbers of nigral and striatal cells are cotransplanted, enhanced DA cell survival is obtained.[13] However, low DA cell survival results when a relatively low number

of embryonic striatal cells are cotransplanted, possibly due to competition among the DA neurons for the limited amount of trophic support provided by the cotransplanted striatal cells.[13]

In addition to a higher proportion of TH^+ neurons in medial dissections of VM, we also found that a higher proportion of these DA neurons expressed aldehyde dehydrogenase (AHD), a retinoic acid-generating enzyme.[33,34] Dopamine neurons of the midbrain can be divided into subpopulations based upon expression of neuropeptides and enzymes,[40] and AHD is expressed in this subpopulation of DA neurons early in development,[41,42] shortly after the appearance of TH.[32,43] The retinoid-synthesizing actions of AHD may play a role in development of DA neurons: Mice deficient in the nuclear receptor Nurr-1, which promotes signaling through heterodimerization with a 9-cis-retinoic acid receptor,[44] failed to produce midbrain DA neurons.[42] In addition, there is a prevalence of AHD-containing neurons among the SNc DA population which project to the dorsal-lateral and rostral regions of the striatum, previously shown to be regions involved in functional recovery after grafting of VM tissue (see below: Regional and phenotypic specification of dopamine neurons).[45]

Inducing Stem Cells and Other Progenitor Cells

The quest for an unlimited cell source for DA transplantation has yielded not only a wide array of potential cells for this use, but also has provided information on the development of DA phenotypes. Totipotent stem cells, adrenal medulla and peripheral nerve cotransplants, carotid body cell aggregates, testis-derived sertoli cells, and cells obtained from transgenic animals are currently being analyzed both for future clinical use and for probing developmental questions.

Pluripotent cells, used both in studies of neural differentiation and as future therapeutic tools, consist of growth factor-expanded neural progenitors, immortalized cell lines, embryonal carcinoma cells, and embryonic stem cells. Growth factor-expanded cells have been transplanted into the adult brain, forming small grafts which exhibit some migration of cells away from the implantation site.[46-48] After growth-factor expanded stem cells isolated from developing CS were transplanted into adult striatum, Svendsen et al observed low cell survival within small grafts, with few differentiated cells expressing neuronal markers.[47] Immortalized cell lines also exhibit a capacity to differentiate into a number of region-specific neuronal morphologies when transplanted into brain (for review, see ref. 49). The transplantation of these cells into neonatal brain resulted in differentiation into neurons and glia with region-specific morphology.[50-53] However, when transplanted into adult brain, Lundberg et al observed that the plasticity of immortalized cells (generated from embryonic striatum or hippocampus) was more restricted: A majority of these cells differentiated into glia in the adult environment.[54] Embryonic carcinoma cell lines can differentiate into terminal, nonproliferating neural phenotypes after pretreatment with retinoic acid and subsequent intracerebral transplantation, [55-58] and in some cases produce TH^+ cells.[56,57] Kleppner et al[55] observed differentiated neuron-like cells which exhibited different patterns of innervation into the host brain depending on the region of the mouse brain in which they were implanted.

Our laboratory has utilized transplantation as a means to investigate DA neuron development: We tested the potential of blastocyst-derived embryonic stem (ES) cells to differentiate into DA neurons.[59] These totipotent cells were transplanted into adult mouse striatum and adult DA-lesioned striatum. The grafts developed large numbers of cells exhibiting neuronal morphology and immunoreactivity for neurofilament, neuron specific enolase, TH, and 5-hydroxytryptamine (Fig. 8.2). Though graft size and histology were variable, typical grafts of 5-10 mm^3 contained 10-20,000 TH^+ neurons, whereas dopamine-β-hydroxylase$^+$ cells were rare. Most grafts also included non-neuronal regions,

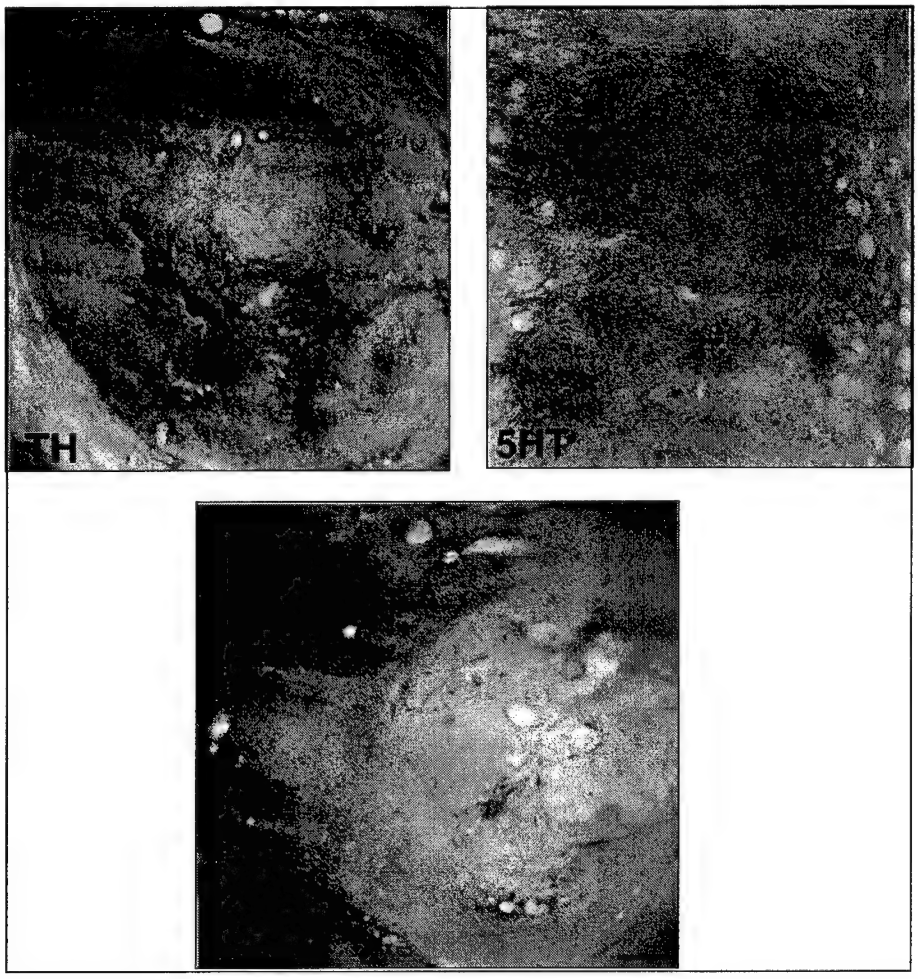

Fig. 8.2. Phenotype of blastocyst-derived embryonic stem cells after transplantation to rat striatum. The grafts develop large numbers of cells exhibiting neuronal morphology and immunoreactivity for TH and 5-hydroxytryptamine (5HT). Dopamine-β-hydroxylase$^+$ cells are rare. Scale bar, 200μm. Reprinted with permission from Experimental Neurology 1998; 149:28-41, ©Academic Press.

immunoreactive for glial fibrillary acidic protein. Both monoaminergic neuronal cell types extended axons into the graft and into the surrounding host brain: TH$^+$ graft axons grew preferentially into gray matter of the DA-denervated rat striatum, as is typical of endogenous striatal DA innervation. This specific innervation pattern has also been observed for DA axons growing into the host striatum from fetal ventral mesencephalic grafts, but is not exhibited by non-DA fibers from the same grafts,[60,61] nor by axons from fetal cortical or striatal grafts to adult striatum.[60] In contrast, 5-HT$^+$ axons from ES cell grafts extended equally into white and gray matter regions of the host striatum. Thus, the difference between

growth patterns of TH^+ and 5-HT^+ axons reflects characteristics that are typical of these mature CNS cell types.

In contrast to results with other cell lines, the ES cells in our study did not seem to be dependent upon the site of transplantation for differentiation into neurons: We transplanted ES cells into mouse kidney capsule to determine the influence that brain-specific environment may have on the differentiation of these totipotent cells. Kidney capsule grafts also developed large numbers of cells exhibiting neuronal morphology and immunoreactivity for neurofilament, neuron specific enolase, TH, 5-HT, and glial fibrillary acidic protein. Neural induction regardless of transplant site in our paradigm is consistent with recent evidence suggesting that neuralization is a default pathway, and occurs spontaneously if pregastrula cells do not receive other inducing signals to form epidermal, mesodermal, or endodermal cells.[62] This was first suggested by experiments showing that cells of the early gastrula ectodermal animal cap, that normally develop into epidermal tissue, all form neural tissue if dissociated.[63] Bone morphogenic protein (BMP-4) and activin have been implicated as the major inducers of epidermal differentiation during gastrulation. Ectopic application of BMP-4 is sufficient to induce epiderm formation in dissociated animal pole cap cells,[64] and homozygous knockout mice lacking functional BMP receptor (BMPR1) die in gastrulation,[65] a time when epidermis would otherwise form. Also, antagonists of BMP-4 or activin signaling, such as noggin, follistatin, and chordin, which are produced in the Spemann organizer region, can induce the ectopic formation of neural tissue.[66-68] Thus, any manipulation that disrupts these epidermis-inducing signals results in neural differentiation. In our experiments, transplanting cells that have been dissociated and expanded at the pre-gastrula stage may disrupt the localized cell-cell communications which otherwise inhibit neuralization. Alternatively, the kidney has been shown to contain many neurotrophic factors and also to enhance the development of TH^+ neurons when cografted with VM.[69] The lack of kidney structure in GDNF knockout mice also suggests the presence of dopaminotrophic factors present in the kidney which may have induced the totipotent ES cells to differentiate along the DA phenotype.[70]

These findings demonstrate that transplantation to the brain or kidney capsule results in a significant fraction of totipotent ES cells developing into putative DA or serotonergic neurons and that, when transplanted to the brain, these neurons are capable of innervating the adult host striatum.

Other Cell Sources

Recent explorations of alternative cell sources have also contributed to the list of cells utilized for the study of DA neuron transplantation. Donor tissue from other species is an attractive alternative to human fetal tissue, particularly from a donor species that breeds in large litters, such as pig. The porcine DA system contains cell groups resembling A8, A9, and A10 of the rat, and differentiate into the homologous cell groups of human.[71] In pig embryos of 28 days, cells of the VM are committed DA neurons expressing TH, yet have not extended processes.[71] When a suspension of fetal pig VM was transplanted into the striatum of immunosuppressed DA-lesioned rats, these animals showed significant reduction in amphetamine-induced rotation, whereas animals not immunosuppressed showed transient behavioral recovery.[6] Behavioral recovery was reversed after animals were removed from cyclosporin, suggesting that the grafts were rejected upon cessation of cyclosporin A treatment.[72] TH^+ neurons were observed within grafts in animals displaying a high degree of rotational correction. In addition to its obvious potential for use in the clinical setting (see below: "Recent clinical progress and developments"), xenotransplantation also provides a tool to analyze the development of various components of transplanted cells, to be discussed below (see below: "The inhibitory environment of adult brain").

Recently, somatic cell cloning, specifically the production of transgenic bovine embryos, has produced an alternative supply of embryonic VM tissue; these VM cells have improved motor function when transplanted into immunosuppressed parkinsonian rats.[73]

In order to circumvent the need for immunosuppression after transplantation, several cell sources have been introduced. Testis-derived sertoli cells have been shown to secrete trophic, tropic, and immunosuppressive factors; culturing these cells with embryonic neurons increased DA neuron survival and outgrowth.[74] Transplantation of these cells produced behavioral recovery in hemiparkinsonian rats, with an increase in TH^+ immunoreactivity in the zone around the transplant.[74] A two month survival period of xenotransplanted porcine sertoli cells into rat brain in the absence of systemic immunosuppression indicates production of sufficient local immunosuppression at the site of transplantation, and may be an alternative method for protecting xenotransplanted cells.[75] Transplantation of chromaffin-like carotid body glomus cells into DA-denervated rat striatum developed into clusters of TH^+ cells with neuronal morphology which extended fibers out into the host striatum, and produced behavioral recovery in turning behavior and sensorimotor orientation three months post-transplantation.[76] And finally, revisiting the adrenal medulla grafts, animal studies utilizing co-grafts of adrenal medulla and peripheral nerve have indicated that this procedure can overcome the major problems encountered with adrenal grafts alone, such as limited survival and transient behavioral effects.[77] Attachment of human fetal VM cells to microcarriers (Cytodex) allowed xenotransplantation into rat host without the need for immunosuppression; however, there was no evidence of TH fiber outgrowth into host striatum, and no functional results were reported.[78]

Regulation of Axonal Outgrowth from Dopamine Grafts

The ability of fetal neurons to be placed into an ectopic region of an adult brain, survive, and extend neurites within this region is remarkable. The functional effects of VM transplants into DA-depleted striatum is often correlated with degree of striatal reinnervation.[4, 13] However, there is some limitation in the ability of the transplanted neurons to extend neurites in the mature brain. Even though the graft-induced elevations in tissue DA concentrations are substantial,[79] values taken distant from the graft suggest that reinnervation of the whole striatum does not occur. The hypothesis for this sharp decline in density of TH^+ fiber outgrowth is that age-dependent characteristics within the host brain alter outgrowth, since extensive outgrowth can be achieved when transplanted into immature (neonatal) host brain. Expression levels and patterns of adhesion molecules expressed by mature host brain are thought to be the culprits of this innervation-inhibitory effect.

The Inhibitory Environment of Adult Brain

The limited regeneration in adult CNS and limited ability of nigral neurons to extend neurites in the mature host brain is also thought to be related to suboptimal properties of the mature striatum as a substrate for the extension of DA neurites.[80] Allografts into immature host brain show robust neuronal and glial migration away from the transplant site, and a high degree of integration and target-directed neurite outgrowth (Fig. 8.3).[3] Fetal cells transplanted into mature brains show neuronal reaggregation around the implant site and limited axonal outgrowth into host brain, suggesting an age-dependent increase in inhibitory or decrease in growth-promoting processes. Since both promoting and repulsive activities influence axonal guidance and extension, alteration of the host brain "substrate" has been examined to obtain more extensive outgrowth from grafts.

The cell-adhesion molecules (CAMs) are involved in promoting neurite extension, by their incorporation into the extracellular matrix and subsequent binding to cell surfaces.[81] Neurite outgrowth from fetal VM cells in culture is enhanced when plated on various cell

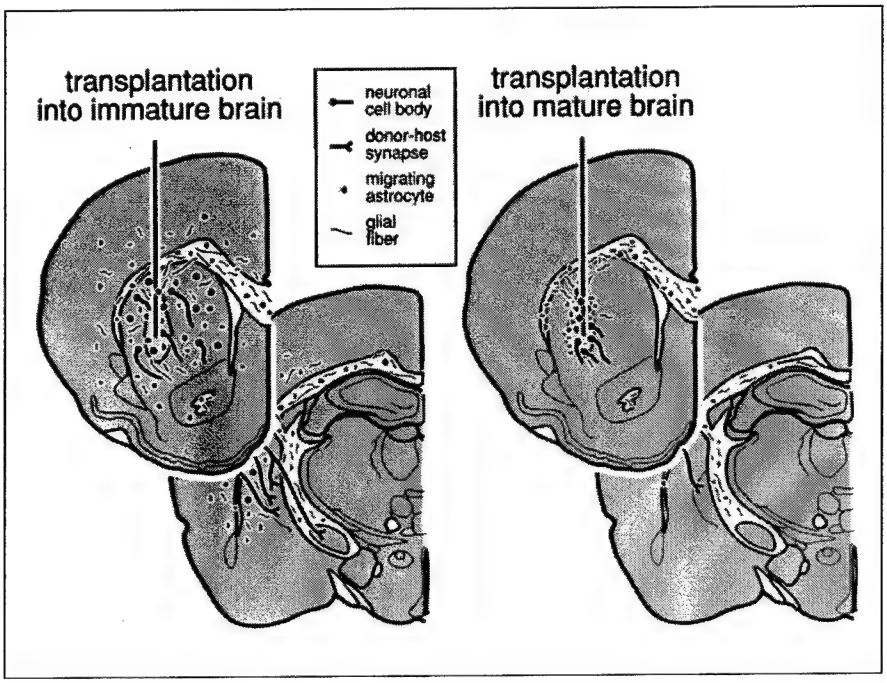

Fig. 8.3. Integration and axonal outgrowth from fetal grafts. Fetal cells transplanted into immature host brain show robust neuronal (black) and glial (grey) migration away from the transplant site, and a high degree of integration and target-directed neurite outgrowth. Fetal cells transplanted into mature brain remain around the implant site and exhibit limited axonal outgrowth into host brain, suggesting an age-dependent increase in inhibitory or decrease in growth-promoting processes. Reprinted with permission from Trends in Neurosciences 1997; 20:477-482, ©Elsevier.

adhesion molecules.[80] Intranigral transplants of VM do not successfully reinnervate striatum unless axonal growth is provoked by bridging the striatum and transplanted tissue via peripheral nerve tissue,[82] laminin track,[83] striatal cells,[84] or grafts of fibroblast growth factor-transfected schwannoma cells.[85] The transient expression of these molecules (GAP-43, NCAM and L1) within VM grafts only during the phase of axon elongation further suggests their contribution to outgrowth of developing neurons.[86,87] Studies utilizing antibodies against growth-inhibiting factors, such as the IN-1 antibody raised against myelin-associated neurite growth inhibitor NT-35/250, have shown enhanced innervation of fetal neocortical innervation into adult host brain.[88]

The argument against outgrowth-inhibitory properties of adult brain stems from studies showing long-distance and target-specific axonal growth from human embryonic transplants into adult rat brain,[89] as well as from porcine embryonic transplants into adult rat brain.[61] The species-specific markers used in our studies of fetal porcine transplants into adult immunosuppressed rat brain allowed comparison of donor glial fiber and donor axonal growth in different host brain regions, demonstrating their distinct trophic characteristics. Target zones in adult host gray matter were selectively innervated by embryonic donor axons

normally destined to form synapses there, whereas donor glial fibers grew irrespective of any target orientation within white matter tracts (Fig. 8.4A).[61] Neuronal axons branched profusely in gray matter target region and only rarely penetrated or crossed white matter tracts. TH^+ fibers from transplants placed into the SN were found coursing up toward the striatum through myelinated fiber bundles, then branching into host gray matter, as also shown by Wictorin from human xenotransplants into rat SN.[89] Interestingly, the non-DA VM cells also grew toward distant gray matter target zones, such as medio-dorsal and ventral anterior thalamus. These data suggest that directional cues for axons, whether diffusible or substrate-bound, are provided by adult host target regions. Since porcine neural development continues four to five times longer than mouse, these axons may develop over a longer time course than that seen in rat-to-rat studies, as illustrated in the development of functional recovery in porcine-transplant recipients (8 weeks post-transplantation) as compared with allografts (6 weeks post-transplantation),

Another aspect of host brain environment which can influence outgrowth from DA grafts is the lesion status: Denervation of the host does seem to promote fiber outgrowth, but has little effect on their survival. Two hypotheses have evolved to explain the apparent increase in outgrowth from grafted cells when transplanted into lesioned versus intact brain: First, outgrowth may be limited due to availability of sites for synaptic contacts, which are increased soon after lesion;[90] alternatively, injury-induced neurotrophic factors have also been suggested,[91] since graft development is enhanced after administration of extracts from injured brain into the implant site.[92] Zhou and Chang[93] showed a bridging method created by a "trophic track" formed by low-dose injection of excitatory amino acids; they observed TH^+ fibers streaming along this lesion track from the intranigral VM graft into the striatum, and hypothesize that the lesion induces trophic molecules, extracellular matrices, and vasculature which support reinnervation by TH^+ fibers.

Regional and Phenotrypic Specification of Dopamine Neurons

Different regions of the striatum are associated with specific behaviors in rat; the dorsal striatum receives primary afferents from the motor areas of neocortex, and has been shown to be preferentially involved in rotational recovery after DA neuron transplantation.[45] In the intact rat, the subpopulation of nigral DA neurons from A9 SNc which co-express AHD project their axons to the dorsal-lateral and rostral regions of the striatum (Fig. 8.4B). As described above (see above: "Utilizing medial VM for dopamine grafts"), the enriched population of DA neurons obtained from a medial versus lateral VM dissection also preferentially expresses AHD; when transplanted into adult DA-denervated rat striatum, these AHD/TH neurons innervate this region of the DA-depleted striatum[3,34] (Fig. 8.4C), showing a preferential reinnervation of the dorso-lateral striatum corresponding to the normal projection pattern of AHD/TH neurons. Specific innervation by subsets of transplanted DA neurons was also demonstrated by Schultzberg, revealing reinnervation of the DA-depleted striatum by the population of grafted VM neurons lacking cholecystokinin (CCK).[40] The CCK^+ fibers were found in a narrow zone immediately adjoining the graft. These data suggest the presence of mechanisms which selectively favor the ingrowth of fibers from the appropriate DA neuronal subset. Thus, enrichment of the DA neuron subpopulation which specifically expresses AHD may allow more appropriate reinnervation of striatum after transplantation, and influence the degree of functional recovery in PD, possibly defined by tropic mechanisms intrinsic to the host brain.

Reconstructing Synaptic Connections with Dopamine Grafts

Functional effects of intrastriatal grafts of fetal DA cells have been illustrated in a range of animal behavioral tests.[94-96] The behavioral effects observed are dependent on the survival of DA neurons within the striatum, since grafting of other tissue produces no behavioral effects[97,98] and removal of transplanted tissue[99] or immune rejection of transplanted functional analyses of DA grafts.

Many groups have used unilateral, intraparenchymal injection of 6-hydroxydopamine (6-OHDA) as the means of producing a unilateral DA denervation of the striatum, then transplanting DA neurons into this denervated striatum. The spontaneous behavior induced by lesion is improved as tested in several parameters, and depends on graft placement, cell number, and density of reinnervation. Due to the imbalance in DA after unilateral lesion, the animal begins to rotate in response to DA-releasing drugs such as amphetamine.[101] The transplantation of DA cells and subsequent reinnervation of the denervated striatum causes the animal to decrease its rotations in response to amphetamine,[102] thus reversing the lesion-induced behavioral abnormalities. Compensation of other lesion-induced changes, such as lesion-induced increases in DA receptor binding,[103] increased levels of enkephalin, and decreased levels of substance P[104] demonstrate the capacity of these DA cells to affect postsynaptic and presynaptic mechanisms.[4,102] However, more complex movements (such as food pellet retrieval, stair case and stepping tests) have exhibited limited responses to DA transplants.[105,106] A microtransplantation procedure which increases the area of striatal reinnervation has shown improved paw reaching in addition to greater striatal reinnervation,[15] suggesting that the limited behavioral recovery of some complex movements so often seen in previous studies may be due to inadequate striatal reinnervation.

Regulated DA Release from Fetal DA Grafts

Methods to improve the number of DA cells that survive transplantation, and enhance the area of the striatum which becomes reinnervated by these cells, are continually being tested; however, the most important factor in obtaining complete and sustained functional effects is the successful formation of synapses between the transplanted cells and the host brain. The use of autologous adrenal cells, fibroblasts transfected with DA-producing enzymes, and other non-neuronal cell types which can secrete DA can perhaps circumvent the problems of limited availability and ethical issues associated with the use of fetal DA neurons. However, functional analyses from these studies indicate that placing a "DA pump" into the striatum may not be as effective in ameliorating the motor symptom of PD as the regulated, synaptic release obtained with transplanted DA neurons;[10] in fact, when DA is directly administered into the ventricle of PD patients, serious psychoses develop,[107] and recent data from differential display has shown the abnormal upregulation of over 10 genes within the striatum.[108] Complications associated with unregulated DA levels are obvious when observing effects of long term L-dopa administration: As PD progresses and the DA neuron degeneration continues, the unregulated formation of DA within the striatum can lead to motor abnormalities such as dyskinesias. The physiological incorporation and regulation of DA release can only be achieved by DA neurons themselves, or by cells which express the complete set of feedback elements required to regulate the release and uptake of DA.

Embryonic DA neurons are not "designed" to produce new connections with mature, established striatal neurons. However, synaptic connections between transplanted VM cells and host cells, as well as afferents from host neurons to transplanted cells, have been illustrated.[109,110] The inclusion of fetal striatal tissue, specifically lateral ganglionic eminence, within VM transplants not only produced increased DA cell survival and extent of reinnervation into the DA-depleted host striatum, but also showed an increased number of

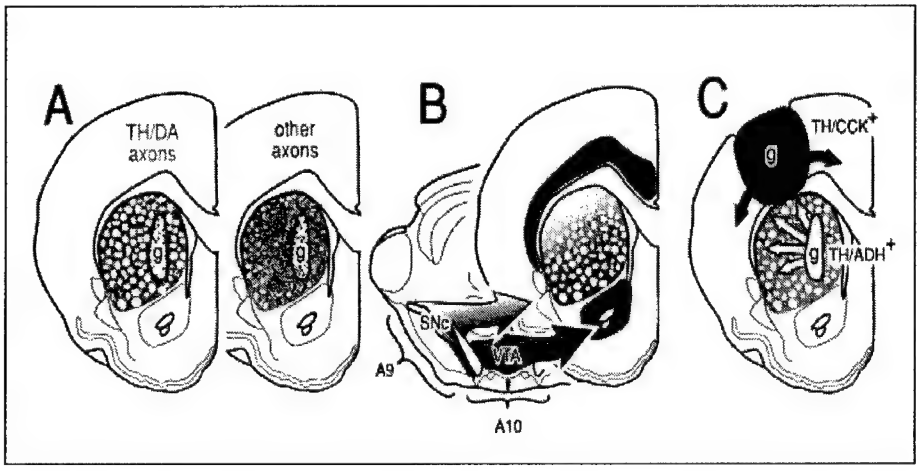

Fig. 8.4. Target-specific innervation by grafted fetal cells. (A) Target zones in adult host gray matter are selectively innervated by embryonic pig donor DA axons normally destined to form synapses there, whereas non-DA donor fibers grow into host myelinated bundles. (B) In the intact rat, the subpopulation of nigral DA neurons from A9 SNc, which coexpress AHD, project their axons to the gray matter of dorso-lateral regions of the striatum. The ventral tegmental area (VTA) neurons from A10 coexpress CCK, and project to ventromedial striatum, nucleus accumbens, neocortex and limbic regions. (C) When the enriched population of TH/AHD neurons obtained from a medial (versus lateral) VM dissection is transplanted into DA-lesioned adult rat striatum, these neurons preferentially reinnervate their normal dorso-lateral striatal target, shown to be involved in rotational recovery after DA neuron transplantation. TH/CCK neurons from VM show different patterns of outgrowth when placed into cortex.[40] Reprinted with permission from Trends in Neurosciences 1997; 20:477-482, ©Elsevier.

host striatal cells which induced the immediate-early gene c-*fos*, indicating a higher degree of host cell activation.[13]

Several studies have shown normalized activity throughout the basal ganglia after transplantation. Nakao et al[111] utilized cytochrome oxidase histochemistry to quantify neuronal activity in the 6-OHDA-lesioned rat; the lesion-induced increases in activity of the entopeduncular nucleus and SN reticulata were reversed by intrastriatal VM grafts, whereas the lesion-induced increases in globus pallidus and subthalamic nucleus were not affected by grafting.[111] The same technique has been used in MPTP-treated monkey receiving VM transplants as well. Dopaminergic grafts increased the metabolic activity of the implanted striatum, particularly in the region of grafts containing greater numbers of DA neurons.[112] Also of interest is the finding that the DA neurons exhibit the highest rate of metabolic activity among all cell types contained in the VM grafts.[112] Positron emission tomography (PET) and carbon-11 labeled 2B-carbomethoxy-3B-(4-fluorophenyl)tropane (11C-CFT) have been utilized as markers for striatal presynaptic DA transporters in a unilateral lesion model in rat. In the lesioned striatum, the binding ratio was reduced to 15% to 35% of the intact side. After DA neuronal transplantation, behavioral recovery occurred only after the 11C-CFT binding ratio had increased to 75% to 85% of the intact side, revealing a threshold for functional recovery in the lesioned nigrostriatal system after neural transplantation.[98]

Autoregulation of DA release and metabolism by intrastriatal grafts has been shown by microdialysis. Treatment with the DA receptor agonist apomorphine produced a decrease in DA in the grafted striatum.[6,113] Correct regulation of DA levels by transplanted striatum is also suggested by the observation that behavioral recovery plateaus at high levels of cell survival, with further neuronal survival providing no additional behavioral effects.[113] Further evidence for the formation of functional synapses and autoregulation from transplanted fetal neurons comes from the observation that dyskinesias, expressed either as contraversive circling after repeated L-dopa injections in rodents[114] or L-dopa-induced dyskinesias in monkey (Widner H, personal communication) are reduced after transplantation. In patients, however, more variable results have been observed.[115,116] These data suggest that even a high amount of extracellular DA within the grafted striatum (either through improved cell survival, increased DA release, or addition of L-dopa) will be regulated in a physiological manner by the transplanted DA neurons.

A more recent approach manipulates the striatal cells themselves to produce L-dopa or DA. Various vector systems have been used to deliver TH, GTP-cyclohydrolase I (the rate-limiting enzyme for tetrahydrobiopterin synthesis), and aromatic L-amino acid decarboxylase to striatal neurons and glia, essentially turning them into DA-producing cells.[117-121] While these approaches bypass the requirement for synapse formation between transplanted VM neurons and striatal neurons, and apomorphine-induced rotation has been decreased in unilaterally-lesioned animals in some studies, the release of DA from these striatal neurons has not been shown to be regulated, and the normal phenotype of these striatal neurons may also be altered. In addition, the drawbacks typically encountered with gene transfer (such as low transduction rates and limited gene expression) also arise.

Clinical Relevance

The Urgency for an Improved Therapy for Parkinson's Disease

The simple concept of replacing lost neurons by inserting new cells was introduced as a new therapeutic strategy for treatment of PD two decades ago. The progressive loss of DA neurons in the SN and resultant decrease of DA levels in its targets produce the signs of PD: tremors, akinesias, muscle rigidity, and postural instability. Although the cause of this cell loss is not understood, therapeutic strategies to correct the impaired motor function due to the unbalanced basal ganglia circuitry have included pharmacological (L-dopa) as well as surgical (pallidotomy,[122,123] thalamotomy,[124] subthalamic nucleus stimulation[125]) approaches. A major obstacle with long term L-dopa treatment is the appearance of severe side effects such as "on-off" phenomena and dyskinesias. Although capable of relieving some parkinsonian motor symptoms (notably tremor and dyskinesias), the surgical approaches each have potential complications, such as cognitive disorders after thalamotomy[126] and hemiparesis, frontal lobe syndromes and hemorrhage after pallidotomy.[122,123]

The specificity of cellular degeneration which occurs in PD (DA neurons of the SN), as well as the well defined target of these degenerating cells (the caudate and putamen), have contributed to the direct application of neural transplantation for this disorder. Early clinical transplantation studies involved the use of catecholamine-secreting adrenal medulla cells.[127,128] The variable and transient alleviation of symptoms, as well as the poor adrenal medulla graft survival and high morbidity of patients, contributed to a transition to fetal cells for PD transplantation. Yet this cell source is not without its drawbacks, specifically the requirement for several fetuses per patient, all within the desired gestation age. However, more recent animal studies utilizing cografts of adrenal medulla and peripheral nerve have indicated that this procedure can overcome the major problems encountered with adrenal grafts alone as discussed above.[77] Several PD patients have received these grafts, and

studies report their success in relieving motor symptoms, through 24 months post-transplantation.[129,130]

Application of Dopamine Transplants to Parkinson's Patients

The successful data from rodent studies with fetal DA cells, as discussed throughout this chapter, were extended to nonhuman primate models of parkinsonism, induced either by the selective DA neurotoxin MPTP or by 6-OHDA.[131] By demonstrating the capacity of these grafts to improve more complex movements and behavioral tasks, and correlating these improvements with histology and PET imaging of the grafts, these studies then led to the first clinical trials in humans. The initial (and in some cases ongoing) issues regarding the technical aspects of the procedure were assessed, such as patient monitoring, implantation technique, donor tissue properties, immunology of allografts, risk assessment of disease transmission, and ethical considerations. The first two patients to be transplanted were middle-aged females each with about a 14 year history of PD, and symptoms consisting of bradykinesia, rigidity, and severe "off" phases.[132] Each patient received four VM regions from aborted fetuses, implanted unilaterally into the caudate and putamen, and were immunosuppressed with cyclosporin. Each showed modest clinical improvement, including improved gait, which lasted several years in one patient and which was lost in the other after 11-13 months,[132] suggesting an immunological rejection of the graft (immunosuppression was withdrawn after 24 months). At nine years, no functional effects persisted, suggesting that cell survival or development was lower than expected.

Examination of the implantation procedure yielded the following improvements: decreasing time between abortion and dissection, and dissection and implantation; buffering of storage/dissection media; adding DNase to final step; reduced cannula size; more tissue. Encouraging results were found with the subsequent two patients receiving fetal VM suspensions: decreasing rigidity, bradykinesia, and number and length of daily "off" periods, which were apparent 6-12 weeks post-transplantation.[133] Three years later, both patients showed near normal 18F-fluordopa uptake in the grafted region, while the contralateral striatum showed decreased uptake when compared with that one year post-transplantation, indicative of progressive degeneration associated with the underlying disease process.[134] A three year study has shown therapeutically valuable improvement in four out of six patients: Rigidity and hypokinesia improved bilaterally; however, no consistent changes in dyskinesias were observed[116] One of these patients was without L-dopa from 32 months and had normal fluorodopa uptake in the grafted putamen at six years.[116] Two additional patients were transplanted utilizing the same protocol, with similar clinical improvements, as well as positive PET results.[135] Bilateral caudate/putamen grafts into two MPTP-exposed patients have produced marked motor improvement in both patients, correlating with increased uptake of fluorodopa.[136] The transplantation of solid pieces of VM have also produced clinical improvement with increased fluorodopa in many (but not all) patients 6 and 46 months post-transplantation.[5,137]

Two instructive clinical studies have provided information on basic parameters. One compared clinical improvements with graft volume: One group of PD patients was transplanted with VM from one to two donors (volume of approximately 20 mm^3), while a second group received tissue from three or more donors (approximate volume of 24 mm^3).[138] Both groups demonstrated significant improvement over presurgical baseline scores; however, the high volume group had significantly greater improvement on all UPDRS scores, suggesting that amount of donor tissue may influence clinical outcome. The second study correlated clinical improvement with immunosuppression: After over two years of immunosuppressive treatment, withdrawal from the cyclosporin treatment produced a decline in

the graft-induced motor improvements, implicating a rejection-induced decline in function.[139]

Recent Clinical Progress and Developments

As mentioned previously (see above: "Other cell sources"), xenotransplantation allows the acquisition of large quantities of accurately aged fetal tissue. The T cell-mediated rejection of xenografts can be inhibited by immune suppression,[140] and studies have shown survival, function, and afferent/efferent connections of xenogeneic cells when transplanted into animal hosts,[6,61] (and see reviews in refs. 141,142). The transplantation of E27 porcine VM unilaterally into the caudate and putamen of twelve immunosuppressed PD patients has produced clinical improvements: UPDRS "off" scores improved 16.9 points in ten evaluable patients at 12 months.[143] One patient from this study died seven months after surgery from a pulmonary embolism; histological analyses using species-specific markers revealed porcine cells and axonal projections from the grafts into host brain. All three identified grafts contained TH^+ neurons (630 TH^+ neurons in all), and non-TH^+ neurons expressing pig-specific neurofilament protein were also observed within, and extending axons out of, the grafts.[8] Microglial and T cell markers showed low reactivity in and around the pig cell graft perimeter.

In addition to this histological study, autopsy data has been published from one other laboratory, who bilaterally transplanted 6.5-9 week human fetal VM into postcommisural putamen of several PD patients. Details from two of these patients who died 18-19 months after surgery of events unrelated to the grafting procedure have been reported.[9,144-146] Both patients showed improved motor function and increases in fluorodopa uptake in the putamen on PET scanning. Histological analysis has shown over 200,000 surviving TH^+ neurons in the male patient (12 sites) which reinnervated over 53% of the right putamen and 23% of the left putamen in a patch-matrix pattern.[9] Electron microscopy revealed axo-dendritic and occasional axo-axonic synapses between graft and host, and analysis of TH mRNA revealed higher expression within the fetal neurons than within the residual host nigral cells.[9] Autopsy of the second patient showed over 130,000 surviving TH^+ neurons, reinnervating 78% of the putamen.[146] Even in these healthy-appearing grafts and a six month regimen of cyclosporin treatment, pan macrophages and T and B cells were observed within the graft sites.[145]

These results have demonstrated the potential usefulness of neuronal replacement therapy for PD, relieving rigidity, akinesia, peak-dose dyskinesias, gait stability, speech, and swallowing. There is no indication that the disease process is negatively affecting the transplanted cells, although the endogenous DA system continues its progressive degeneration.[116,134] The striatum has a remarkable capacity to compensate for very low levels of DA, as evidenced by the lack of parkinsonian symptomology until 80% of DA is lost. Thus a substantial, though perhaps incomplete, reinnervation may allow maximal functional outcome. However, the basic mechanistic problems with these grafts as outlined in the above review, specifically the limited development and reafferentation of host brain by these grafts, require the continued efforts of investigators in this field. Other issues such as graft location,[147] immunologic questions,[145,148] further progression of the disease, and continued exposure of fetal cells to L-dopa, remain under intense investigation.

Conclusion

The current understanding of the normal in situ maturation and phenotypic specializations of DA neurons located in the adult substantia nigra parallels the observations made of the development of committed fetal dopamine neurons placed as grafts into the adult CNS. The molecular signaling necessary for the final morphological specializations and connectivity of the nigro-striatal DA system must therefore be largely intrinsic to the developing DA neurons, or, alternatively, present in significant detail in the adult brain for this process to be completed in a normal way.

These findings may be clinically applied to further improvements in DA neuron "replacement" in the PD brain, and provide functional restitution to patients with neurodegenerative diseases.

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Cellular and Molecular Treatments of Neurological Diseases

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This special issue on cellular and molecular research for novel treatments of neurological disease is the result of the Second Cellular and Molecular Treatments of Neurological Diseases (CMT) Conference held in Cambridge, Massachusetts, at the American Academy of Arts and Sciences in October 1998. The meeting brought together over 150 scientists and students working in the field and approached new treatments through three paradigms. First, neural transplantation as a cell replacement therapy was evaluated. The ground-breaking work of Olle Lindvall and others in the field was described by him and other speakers. The neural transplantation field is now beyond the proof of principle and much work remains to develop and optimize these procedures. The "stone age of neural transplantation" may soon be past; we look forward to many new discoveries and procedural improvements to make this procedure available to many patients with neural degenerative diseases. In this small and novel branch of neuroscience, we find ground-breaking work on very specialized systems that can be repaired by neural cell transplants. Professor Ray Lund described his work on the retinotectal system and how it can be repaired. Dr. Lund and colleagues previously demonstrated neural circuitry replacement in the retinotectal system and at this conference described recent work on photoreceptor layer reconstruction in mice with dystrophic photoreceptors of the retina.

At the Second CMT Conference at the AAAS in Cambridge, Massachusetts, the next two themes involved progenitor cell biology and cell differentiation, as well as gene therapy for neurological diseases. In the progenitor cell biology section, a number of eminent speakers, including Dr. McKay, Dr. van der Kooy, Dr. Whittemore, Dr. Mehler, Dr. Brüstle, and Dr. Svendsen described new ideas and conceptual developments of this field. Dr. Clive Svendsen and Oliver Brüstle demonstrated how progenitor cells can be implanted in various systems and used as therapeutic vehicles.

In the third theme of the conference, gene therapy for neurological diseases, a number of distinguished speakers described their work. The engineering of herpesviral vectors by Dr. Joseph Glorioso was a *tour de force* describing the stepwise approach to improved vector design. Dr. Ron Mandel elaborated on his elegant work on using gene therapy for the treatment of parkinsonism in animal models using recombinant AAV vectors. And finally, Dr. Howard Federoff described advanced vector technology and functional neurobiology necessary for transcriptional activation of posttranscriptional control in gene delivery and gene therapy. These communications at the conference provided a worthwhile learning experience, and we hope that the readers of *Experimental Neurology,* through this special issue, can enjoy some of this work as well.

This special issue begins with a paper by Kordower *et al.* demonstrating that cellular delivery of trophic factors can prevent degeneration typically seen in the striatum of Huntington's disease. These studies are based on findings of extensive neuroprotection caused by a number of factors in the striatum such as NGF, basic FGF, BDNF, and CNTF. The distinction between pharmacological and cellular delivery of factors is also elaborated by Kordower and coauthors. The initial finding of extensive neuroprotection by implanted cells producing NGF in the striatum by Schumacher *et al.* (13) was confirmed by a number of other studies, including genetically engineered progenitor cells producing NGF (12). A number of other studies have also shown that pharmacological treatment with NGF, while creating neuroprotection, was not as effective as the cell-based NGF delivery seen in other work (3, 9, 14). The extensive neuroprotection created by NGF and CNTF (4–7) indicates that the cell-based delivery of neurotrophic factors, while very specific, may also include dynamic receptor inductions, as well as improved efficacy through cofactors. The review by Kordower *et al.* discusses the extensive neuroprotection possible in a variety of animal models with relevance to Huntington's disease.

With regard to another part of the brain, Dr. Lund and colleagues have over a number of years shown us how complex circuitry reconstruction is possible using the transplant paradigm. Using transplantation as a

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tool to investigate the plasticity of the nervous system, the group led by Dr. Lund has determined how adaptive the visual system can be with such procedures. In the current review by Dr. Kwan *et al.,* they demonstrate how the photoreceptor layer of the retina can be reconstructed through implantation of postnatal retina fragments. The dystrophic retinal layer, as seen in retinitis pigmentosa, is therefore a target for their research. The finding of simple light/darkness discrimination in the transplanted mice suggests that there is some functional rebuilding of the circuitry. These authors argue that logical circuits that are typical of peripheral nerve stimulation in the retina may also be a therapeutic target of future transplantation work.

The review by Wolfe *et al.* describes the steps necessary to engineer one class of viruses, the herpes simplex viruses (HSV), as vehicles for gene delivery and gene therapy. The systematic approach of determining the biology of the virus by these investigators illustrates that understanding of the genome is necessary prior to accomplishing effective gene delivery. In particular, the HSV vectors have been reengineered to control and use the latency promoter and regulatory regions. Moreover, in the elimination of toxic and replication-competent sequences, the HSV has been structured to maximize its transgene-carrying capacity while not eliminating its specific neurotropic characteristics. The experiments were designed to address the cell-targeting issues necessary for effective gene therapy in the CNS. Wolfe *et al.* also evaluate and discuss the neurodegenerative applications of such viruses as well as the potential application to cancer conditions (15, 16).

The recent work by Mandel *et al.* (10, 11) has demonstrated important new steps toward functional gene delivery in the striatum involving both dopamine-related production and neurotransmission, as well as neuroprotection first described by Bohn and Choi-Lundberg (1). The review reasons that the eventual engineering and development of viral vectors may provide a more continuous release of drugs in the brain. This obviously is still an unaccomplished goal but Mandel *et al.* argue that some of their work on recombinant engineering of adeno-associated viruses illustrates this point. Mandel and colleagues have shown that the co-infection of MD vectors containing the tyrosine hydroxylase gene, as well as the cofactor producing GTP cyclohydrolase, can improve the production of L-dopa in striatum through co-infection of cells. They demonstrated gene expression for up to 6 months. This work forms a data base for future studies toward more effective gene delivery and gene therapy paradigms in that system.

In Halterman and Federoff's work in this special issue, neurodegeneration caused by ischemic conditions is discussed. Their work elaborates on the molecular mechanisms involved in this damage and demonstrates that the p53 transcriptional activators, as well as the PAS family proteins (here, HIF-1α), can drive ischemia-induced changes toward degeneration of neurons in the brain. Their data and thinking support the idea that the pathologic gene expression seen under stressors can be modified by adaptive gene expression toward neuronal protection. In particular, Halterman and Federoff elucidate gene delivery as a tool in cell culture and *in vivo* applications to modify gene expression or pro-death gene expression (2, 8).

The CMT Conference at the American Academy of Arts and Sciences was an academic initiative through the Departments of Neurology and Psychiatry at Harvard and included a number of eminent lecturers and was organized by Drs. Ole Isacson and Xandra Breakefield. We hope that this conference series will continue and look forward to the next meeting in the year 2001. Meanwhile, the pages available in this forum of *Experimental Neurology* will surely provide communication on these and related issues.

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The Potentials of Gene Therapy for Treatment of Parkinson's Disease

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Development of molecular biological techniques has resulted in discovery of genetic mutations that are responsible for various neurodegenerative disorders including Parkinson's disease (PD) and these discoveries are leading to better understanding of the pathogenesis of these disorders [1,2,3]. Such technologies also lead to development of genetic engineering methods as potential therapeutic modalities for these disorders [4-6]. This combination of development anticipates new treatments for these disorders that have not been amenable to traditional therapies. The idea of gene therapy has been proposed decades ago, but a successful clinical application has not yet been implemented [4]. As with most of the scientific developments, the initial implementation of ideas reveals unanticipated problems and further improvements and modifications follow. This chapter will discuss the basic concepts and methodology of gene therapy including recent promising advances and limitations of the current techniques. The potential of the gene therapy for PD will then be illustrated through specific examples in animal models of PD.

I. Basic concepts of gene therapy

In its simplest concept, gene therapy delivers DNA materials into host instead of the final desired product, utilizing the internal machinery to transcribe the DNA information into RNA, then protein, which in turn, may produce a desired product. Delivery of DNA provides more efficient, sustained, and localized supply of the product than delivering the product itself. The defective function of abnormal genes in genetic disorders could be complemented by normal genetic information. In cases where the genetic abnormality leads to gain of toxic function, a good understanding of the mechanism of toxicity is necessary in order to intervene and neutralize the toxic function. In many cases, gene therapy does not directly target the genetic mutations, but rather provides a biological minipump that delivers pharmacological compounds directly into the localized sites of the body [7].

II. Gene therapy methods for the nervous system

Two general approaches have been employed for delivering therapeutic genes into target tissues: the *ex vivo* gene therapy by which cells are genetically modified *in vitro*, then transplanted into the host, and the *in vivo* gene therapy by which therapeutic gene is introduced directly into the host somatic cells *in situ*, using viral vectors (Fig. 1).

1) **Ex vivo *modality***

For *ex vivo* gene transfer, the cells to be transduced with the genes should be easily obtainable, readily cultured, able to express the transgenes, and undergo the selection process that is used to enrich the population of cells transduced. The cells should be non-oncogenic, immunologically compatible with the recipient, and survive well in the brain. The cells then could be genetically modified to secrete the neuroactive substances such as neurotransmitters and neurotrophic factors and serve as a biological pump at a localized site in the brain. Primary skin fibroblast cells taken from adult animals satisfy most of the criteria outline above. Fibroblasts could be easily obtained from patients: their own skin biopsy could be modified into customized immunocompatible donor cells. The astrocyte is also an attractive cell type for grafting studies in the CNS due to its intrinsic supportive role in the CNS. These cells are, however, not as easy to transduce with retroviruses as fibroblasts [8].

The ideal cells for CNS somatic gene therapy would be cells of CNS origin with neuronal features such as storage mechanism, secretory pathways, and regulatory signal transduction pathways for the final product. Primary neurons could be genetically modified to boost their survival or alter their phenotypes. To avoid obtaining primary neurons each time, while reducing the risk of tumor formation, techniques of conditional immortalization using non-transforming oncogenes have been employed[9,10]. More recently, CNS progenitor cells that are capable of cell division have been isolated from fetal and adult brains, particularly from regions that undergo neurogenesis beyond the developmental ages such as subventricular zone, olfactory system and hippocampus [11], but also from other areas of adult brain [12]. These were propagated in the presence of epidermal growth factor [13] or fibroblast growth factor [11]. These cells can then differentiate into glial or neural phenotypes in culture and in the brain after transplantation [11,14]. Understanding proper differentiation signals for the desired neuronal phenotypes would provide a means of generating unlimited supply of transplant donor cells. For example, Nurr1 is a critical transcriptional factor that is important in development of dopaminergic neurons [15,16]. Nurr1 has been shown to induce a dopaminergic phenotype, mainly the expression of the first and rate-limiting step of dopamine synthesis, tyrosine hydroxylase (TH) in neuronal precursor cells [17]. However, whether Nurr1 alone is sufficient to induce full-fledged dopaminergic

phenotypic expression is not clear. Meanwhile, modifying progenitor cells by additional neurotransmitter synthesizing genes, such as TH may be necessary [18,19].

The advantages of *ex vivo* gene transfer include the ability to control and monitor the gene transfer process before the cells are placed back into the subjects. The biochemical effect of the transgene can be characterized and potential tumorigenesis can be assessed. Toxicity of the virus can be screened. In addition, grafted cells may provide useful functions beyond what is provided by the transgene, such as serving as a substrate for axonal growth, or restoring synaptic contacts with the host neurons. However, for certain applications, direct *in vivo* gene therapy may be more suitable.

2)* In vivo *or direct gene transfer

Host CNS cells may be better equipped to produce the desired products from the genetic information than foreign donor cells since they may possess the machinery necessary for the post-translational modification of the gene products, the appropriate cofactors, and the ability to secrete the neurotransmitters or other neuroactive compounds. Viral solutions may also be less intrusive than grafts to the normal brain physiology because grafts of genetically engineered cells could disrupt the normal architecture. Moreover, grafting genetically modified cells is more suitable for delivery of secretable products that act on cell surface receptors such as neurotransmitters and neurotrophic factors, but not for delivery of intracellular proteins such as protein kinases, receptors and transporters. On the other hand, the consequence of delivering therapeutic molecules (e.g., dopamine) directly into host cells (e.g., striatal neurons) that does not normally express such molecules is not as predictable as delivering them extracellularly by grafts serving as a biological minipump. Expression of the gene product could lead to untoward alteration of the host cell function. The major disadvantages of *in vivo* gene transfer also include the safety of using viruses directly. Use of neurotrophic viruses such as herpes virus, adenovirus, adeno-associated virus, and lentivirus has recently been shown to be effective in gene transfer into neurons.

3) Vector constructs

Although many viruses have been explored as vector systems, the following virus vectors have been most extensively applied to CNS gene transfer. Each has its own advantages and disadvantages and there does not seem to be a clear universal vector system of choice at this point. A simplified general scheme of virus vectors is described in Fig. 2.

a) Neurotrophic virus vectors for in vivo gene therapy

Herpes simplex type I virus (HSV-1) vectors infect a wide range of host cells including post-mitotic neurons and can establish latency indefinitely within the neuron. Two general approaches include 1) use of recombinant HSV with various deletions that render the virus replication-defective [20,21] and 2) use of an amplicon based on plasmid vectors containing the transgene plus minimal HSV genes such as origin of DNA replication and packaging site. Because of the large size of HSV, development of safe vectors will require extensive understanding of the genome to be able to reduce the neurovirulence and cytotoxicity [22,23]. On the other hand, the large size of HSV viral genome (152 kilobase pairs [kb]) also allows insertion of a large foreign DNA, which may prove very useful for certain *in vivo* CNS gene therapy.

The adenovirus represents an attractive candidate for direct gene transfer into the CNS because of its high titer virus stocks, efficient infection of post-mitotic cells, and the relatively benign nature of the virus infection [24]. The adenovirus is a double-stranded, linear DNA virus that, in its wild-type form, causes a variety of mild flu-like ailments. The potential for adenovirus vectors to achieve efficient gene transfer to neurons, microglial cells, and astrocytes *in vivo* have been noted [25,26]. Expression of the transgene products is limited in duration, probably due to the immune reaction from the host [27,28]. Recent constructs of adenovirus vectors which delete all viral genes show high levels of stable expression with minimal toxicity and allow inserts of up to 37 kb size [29].

Adeno-associated virus (AAV) is a nonpathogenic DNA virus, which requires helper viruses such as adenovirus and herpes virus for productive infection. AAV vectors have minimal viral sequences of their own and therefore have minimal deleterious consequences. Because of the small genome, AAV can accommodate only about 5 kb inserts. AAV integrates into a specific site in the chromosome 19q13.3 [30], and long-term expression of

various neural genes have been noted in the CNS with minimal host reactions [31-34]. Despite its limited capacity for transgenes, AAV may be the safest vector capable of long-term *in vivo* expression at this point.

b) Retrovirus vectors for ex vivo therapy

Disabled murine leukemia retroviruses have been most widely used for gene therapy, especially for most *ex vivo* applications. Retroviruses infect a broad range of cells with high efficiency. Many generations of modified vectors have been developed to lower the chance of recombination event that can lead to wild type virus generation [35]. The integration of provirus into the chromosome is stable and precise, but the location is apparently random, and therefore could produce an insertional mutation by disrupting a normal gene. Homologous recombination of the retroviral vectors with the retroviral genome in the packaging cells could generate the wild type virus, which can lead to the formation of tumors [36]. However, current generation of packaging cells has minimal overlap of sequences with helper viruses and the helper virus genes are separated into two separate plasmids [37]to further minimize the possibility of recombination. Disadvantages of retroviral vectors include relatively low viral titers, usually less than 10^6 infectious units per ml. The size of the gene that can be inserted is limited to 8 to 10 kb. Retroviruses also require dividing cells with active replication and DNA synthesis for the provirus integration to occur [38]. Therefore, retroviruses are not useful for *in vivo* gene transfer into non-dividing cells, but remains as the mainstay tool for *ex vivo* therapies.

c) Hybrid vectors

Hybrid vectors combine advantageous properties of different virus vectors [39]. A good example of hybrid vectors is lentiviral vector which combines the ability of the human immuno-deficiency virus (HIV-1) to insert a provirus copy into the genome of nondividing cells with wide host range and high infectivity of stomatitis virus G surface glycoprotein. Unlike other retroviruses, lentiviruses can infect non-dividing cells by using nuclear import machinery to gain entry into the nucleus in quiescent cells and then integrating viral DNA into the host chromosome. Stable transgene expression in neurons was noted *in vivo* for up to 3 months without appreciable pathological changes and immune response [40]. Because the HIV prevents cells from undergoing mitosis, development of packaging cell lines that can facilitate vector generation has been hampered. Further safety of the

vectors must be assured before clinical use of this HIV-based vectors can be contemplated. Another example of hybrid viruses is HSV/AAV vector. AAV rep gene and inverted terminal repeats of AAV are incorporated into HSV amplicon vectors to enable amplification and specific integration of desired gene into chromosome [41]. These new generation of hybird vectors may provide ideal tools circumventing the safety issues and achieving long-term expression of the transgenes.

d) Further development of gene therapy vectors

Before these gene therapy methods can be applied to human neurodegenerative disorders, several issues need to be resolved. Safety issues of gene therapy vectors have been discussed above and a great deal of progress has been made in this regard. Another safety issue concerns the transgene itself. Expression of the transgenes and secretion of the transgene products into the serum can lead to immune responses to the transgene products. However, it is not clear whether immune system can recognize the transgene products that are expressed intracellularly but are not secreted.

Another problem of gene therapy has been the inability to obtain long-term stable transgene expression *in vivo* [42]. Long-term expression is easily achieved in cultures throughout many passages of donor cells. Once the cells are implanted in animals, however, transgene expression diminishes rapidly [42]. Although this remains an unresolved problem, several possibilities appear promising. Vectors that lead to integration of the transgenes into the chromosomes are more likely to be stable than vectors that stay episomal. In addition, integration sites utilized by the vectors play a significant role in the transcriptional activity of the transgene. Retroviruses integrate into the chromosome during active division but the integration site may become dormant in the quiescent state that grafted cells assume in vivo. Taking advantage of the ability of lentivirus to enter into the nucleus of non-dividing neurons, and to integrate into sites that are active during quiescence, investigators have been able to achieve long-term expression of transgenes in neurons of the CNS [40]. AAV also integrates into the genome of host neurons and has shown promise in its ability to sustain long-term gene expression *in vivo* [31-33].

Promoter types may also significantly influence long-term expression. Most gene therapy vectors contain viral promoters to express the transgenes, but their transcriptional activity may be suppressed in the somatic cells. Promoters of endogenous cellular genes such as a constitutive housekeeping gene promoters [43] or cell type-specific promoters [44] have had some initial success. However, most tissue-specific promoters have a low basal activity. A more recent approach has shown enhanced transcriptional activity from synthetic promoters engineered from a combination of operator elements [45]. The effect of the promoter may depend on the cell type and vectors used [46,47].

Although the current focus is on achievement of high and stable expression of transgenes, excessive levels of products such as L-DOPA are likely to produce adverse side effects such as dyskinesias. Optimal levels are critical for physiological function of many substances, and the ability to regulate the transgene expression is important. Promoters that can be regulated externally or have a built-in feedback mechanism will be an important advance for the future. One such system was developed to express the transgene by promoters whose activities can be either turned on or off by administration of tetracycline both *in vitro* [10] and *in vivo* [48]. Other combination of nontoxic substances and heterologous transcription factors that bind to regulatable promoters have been developed [49,50].

III. Strategies of gene therapy for Parkinson's disease

1) Dopamine replacement by delivering neurotransmitter synthesizing genes

The pathogenesis of PD is well understood and the efficacy of replacing the neurotransmitter dopamine is well established. However, long-term treatment with the precursor, L-3,4-dihydroxyphenylalanine (L-DOPA) produces dyskinesia and fluctuations in most patients. These problems can be either prevented or reduced by continuous delivery of dopamine into striatum [51]. Various dopamine producing cells including dopaminergic fetal neurons can also provide continuous and site-specific dopamine delivery [52]. However, given the limitations in obtaining proper fetal tissues in sufficient amounts, alternative sources of donor cells such as xenografts and genetically engineered cells have been proposed. To provide L-DOPA into the brain by gene therapy, initial studies have focused on introducing TH gene, which is the first and rate-limiting step of dopamine synthesis, using established cell lines

such as rat fibroblast 208F cells [53], NIH3T3 cells, and endocrine cell lines, AtT-20 and RIN cells [54]. Subsequently, to overcome the problems with the tumor cell lines, primary cells such as fibroblast cells [55], or astrocytes [8] have been shown to produce long-term graft survival without tumor formation or immunological rejection. More recently, neuronal precursor cells have been genetically modified to express dopaminergic phenotype, but their efficacy in *in vivo* models is not clear yet. In addition, direct *in vivo* TH gene transfer into the brain cells using viral vectors has also been attempted [56,57]. Most of these studies have shown partial reversal of apomorphine-induced rotation, which is used as a rodent behavioral model of PD.

Further refinement of the L-DOPA delivery has been achieved by using the gene GTP cyclohydrolase I (GCH1), which is the first and rate-limiting step in the biosynthesis of tetrahydrobiopterin, an essential cofactor for TH. Experimental gene therapy studies have shown that that double transduction with TH and GCH1, by either *ex vivo* [58] or *in vivo* [33] gene transfer, is necessary for sufficient L-DOPA and dopamine production in rat models of PD (Fig. 3A). Microdialysis studies have shown direct biochemical evidence for the efficacy. These studies have also pointed out the limitations of the rotational behavioral model, which have contributed to misleading conclusions of previous studies about the efficacy of using TH gene alone. Importance of GCH1 has been underlined by the finding that mutations in GCH1 in L-DOPA-responsive dystonia (DRD) patients lead to loss of its function to generate cofactor, tetrahydrobiopterin (BH_4) [59]. The absence of cofactor results in the lack of L-DOPA and dopamine production, parkinsonism and dystonia of DRD patients. Interestingly, symptoms of DRD patients can be almost completely ameliorated by L-DOPA therapy without development of long-term complications that is commonly observed in PD. Such an ideal response to L-DOPA in DRD abrogates any need for gene therapy, but provides us with an insight as to the importance of events that occur downstream from production of L-DOPA, in the therapy of PD.

The second step in dopamine synthesis is decarboxylation of L-DOPA to dopamine by aromatic L-amino acid decarboxylase (AADC). Although there seems to be some decarboxylation of L-DOPA to dopamine even with severe dopaminergic neuronal loss in animal models and patients with PD [60,61], the exact source and sites of AADC *in vivo* are not clear [62]. When additional AADC was provided within the genetically modified grafts producing L-

DOPA, the final levels of L-DOPA and dopamine decreased, presumably due to the feedback inhibition of dopamine on TH [63]. Therefore, keeping the cytoplasmic dopamine levels low by sequestering it into the vesicles at high concentrations seems to be a critical strategy of dopamine delivery [64]. Combination of L-DOPA-producing cells with cells that can decarboxylate L-DOPA to dopamine and storage them in vesicles may be more optimal, but this has not been tested directly yet (Fig. 3B).

In the strategies of providing a source of L-DOPA or dopamine as described above, the precise control of the exact amount of L-DOPA or dopamine given by the gene therapy will be critical and may be difficult to achieve. Excessive dopamine will be detrimental to the patients. One could attempt to control the precise levels by using the regulatable promoters described earlier. We proposed another approach of combining the precursor L-DOPA administration with genetic machinery to process L-DOPA more efficiently [65]. Providing additional AADC in the denervated striatum of rat models of PD resulted in higher levels of dopamine produced from L-DOPA [34]. However, increasing the level of dopamine is not as critical as prolonging the duration of elevation and buffering the fluctuating levels of dopamine following administration of L-DOPA. Combined use of AADC for decarboxylation of L-DOPA and vesicular monoamine transporter (VMAT) for efficient storage of dopamine within cells sustained high levels of dopamine from L-DOPA administration compared to using only AADC [64] (Fig. 3C). This underlines the importance of storage and gradual release of dopamine in pathogenesis and treatment of fluctuating responses in PD.

Although site-specific and sustained delivery of dopamine would provide a major advance in PD therapy, it is not clear whether this approach will be sufficient to restore the entire symptom complex of PD including some of the L-DOPA-associated complications such as dyskinesia. The role of nondopaminergic system in PD needs to be explored further. In addition, restoration of neuronal connectivity by the graft and complex functions such as feedback interaction of dopaminergic neurons with the striatal neurons may be important. However, it is not clear whether such proper interactions could be restored even with fetal dopaminergic neurons or dopaminergic neuronal cell lines generated by *ex vivo* gene therapy.

2) Repair and protection strategy by neurotrophic factor delivery

The peptidergic growth factors have wide-ranging effect on the survival of neuronal populations as well as on regulation of neurotransmitter release and modulation of neural activities in the adult CNS. Although the precise mechanism of the neuronal degeneration of PD is not known, neurotrophic factors or growth factors may prevent or slow cell death cascade regardless of the initial triggering event. Several growth factors that have shown to possess trophic activity in the dopaminergic system include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, basic fibroblast growth factor (bFGF), transforming growth factor ß (TGFß), and glial cell line-derived factor (GDNF) [66-71]. All of these factors have been shown to enhance dopamine neuronal cell viability in vitro, although this in some cases appear to be mediated by indirect effects on other cell types [70,72]. In vivo effects have been mostly noted by neurotrophin and GDNF family members. In vivo delivery of BDNF enhances striatal dopamine turnover and decreases nigral dopamine turnover, as well as causes contralateral rotations and locomotor activity in amphetamine treated rats [73,74]. Direct infusion of BDNF above the SN increases the firing rate and even the number of electrically active dopamine neurons [75] in this pivotal brain region. The presence and expression of BDNF and its receptor, TrkB in the adult SN indicates that BDNF may regulate DA function under normal physiological conditions [76,67,77]. As in the developing CNS, striatal BDNF is retrogradely transported to DA neurons in adult brain [78]. Infusion of of NT-4/5 into the ventral mesencephalon also causes increased striatal DA turnover and release and locomotor activation following the amphetamine stimulation [79]. The NT-3 molecule also enhances amphetamine-induced contralateral turning and decreases SN DA turnover [74]. One of the most potent factors for the DA substantia nigra is GDNF. In normal animals, GDNF increases both spontaneous and amphetamine-induced motor behavior. These motor effects occur in parallel with increased DA levels and turnover in the SN, and enhanced DA turnover and consequent reduction in striatal DA levels [80]. Infusion of GDNF into the striatum results in retrograde transport to the SN DA neurons [81].

These results support the concept that even in the adult brain, developmentally dependent growth factors may be able to augment DA neuronal function. The problem with pharmacological delivery of the peptidergic growth factors is access to the brain. The non-oral delivery methods of these substances frequently require methods that circumvent the blood-brain-barrier, for instance by neurosurgical intraparenchymal or intraventricular infusions.

These methods are site-specificic but invasive and often unpredictable. Tissue damage at the cannula placement site is frequently seen. Moreover, effective delivery is limited by diffusion properties of the factor within brain parenchyma [82]. Additionally, intraventricular administration of the TrkB ligand BDNF is ineffective due to binding by truncated TrkB receptors which are present in the ependymal lining of the ventricles.

As an alternative to direct infusion, methods for cell-mediated delivery of therapeutic proteins have been developed including ex vivo as well as in vivo gene transfer. Implants of BDNF-producing fibroblast cells protected against neurotoxins such as 6-hydroxydopamine (6-OHDA) [83] and 1-methyl-4-phenylpyridinium (MPP^+) [84]. In addition, sprouting of dopaminergic fiber has been demonstrated in BDNF-transduced fibroblasts implanted into the midbrain [85]. GDNF has been delivered into rat substantia nigra neurons by adenovirus or AAV vectors and protected these neurons from progressive degeneration induced by 6-OHDA lesion of the dopaminergic terminals in the striatum [26,86]. A more practical approach of injecting the viruses into the striatum resulted in cellular and behavioral protection from striatal 6-OHDA lesions [87,88].

3) Other potential targets of gene therapy for Parkinson's disease: intervention of pathogenesis

Understanding the etiology and pathogenesis of PD may allow us to intervene directly at the level of pathogenesis and forestall the clinical manifestations or stop progression of the disease. Although the etiology of PD is still not known for most cases, recent discoveries of mutations in α-synuclein in families with autosomal dominant inheritance of PD [1,2] and *parkin* in families with autosomal recessive juvenile parkinsonism [3] may provide us new clues. Knowledge of the precise steps by which these mutations lead to dopaminergic neuronal death could allow us to apply these findings to sporadic form(s) of PD as well.

Although the precise strategy awaits further understanding of the mechanism of the toxicity by the genetic mutations, general therapeutic approaches for a known genetic defect can be outlined. For autosomal recessive genetic disorders which commonly confer a "loss of function", augmentation of the missing genetic information may restore the abnormalities. On the other hand, a dominant disorder may involve a "gain of function" induced by the mutant protein. For these disorders, it is not possible to simply replace the defective gene with a normal one.

Techniques for specifically targeting the abnormal sequence and replacing it with a normal sequence exist and are applied in generation of transgenic animals with "knock-out, knock-in" strategy [89]. However, these are not easily applicable to humans. The intervention of gene expression at the level of messenger RNA could be attempted. It may be possible to integrate viral or non-viral vectors carrying catalytic antisense RNAs or ribozymes which bind to, and irreversibly cleave, abnormal mRNAs [90]. Antisense RNA has been used to hamper transcription, processing, transport and/or translation of mRNAs in a variety of cell types.

Intervention further downstream of the abnormal protein expression will be possible once the normal physiology of the mutant protein is known. Pharmacological blockade of the effects of the mutated protein may be envisaged by introducing genes that will produce inhibitory substances. In addition, even in cases where the exact function of the mutant protein in unknown, understanding the general process of dopaminergic cell death could lead to other approaches in preventing disease progression. For example, the genes preventing apoptosis could be expressed in dopaminergic neurons to save them from their demise [91]. Given the possible role of oxidative stress in dopaminergic neuronal degeneration in PD, overexpression of free radical scavenging enzyme, such as superoxide dismutase (SOD) may protect dopaminergic neurons from degeneration. Experimental models show that SOD overexpression protects dopaminergic neurons from the neurotoxicity [92]. In addition, SOD enhances the survival of the grafted neurons [93].

In summary, gene therapy has the potential to provide an efficient delivery of various genes and products into a localized site. Along with advances in new genetic discoveries and understanding of the pathogenesis of PD, this may provide the most efficient means of therapeutic intervention at appropriate levels. In addition, gene therapy experiments contribute to further understanding of the biology of the diseases. Gene therapy is an evolving concept that could bridge the new molecular understanding of the diseases and current modalities of PD therapy.

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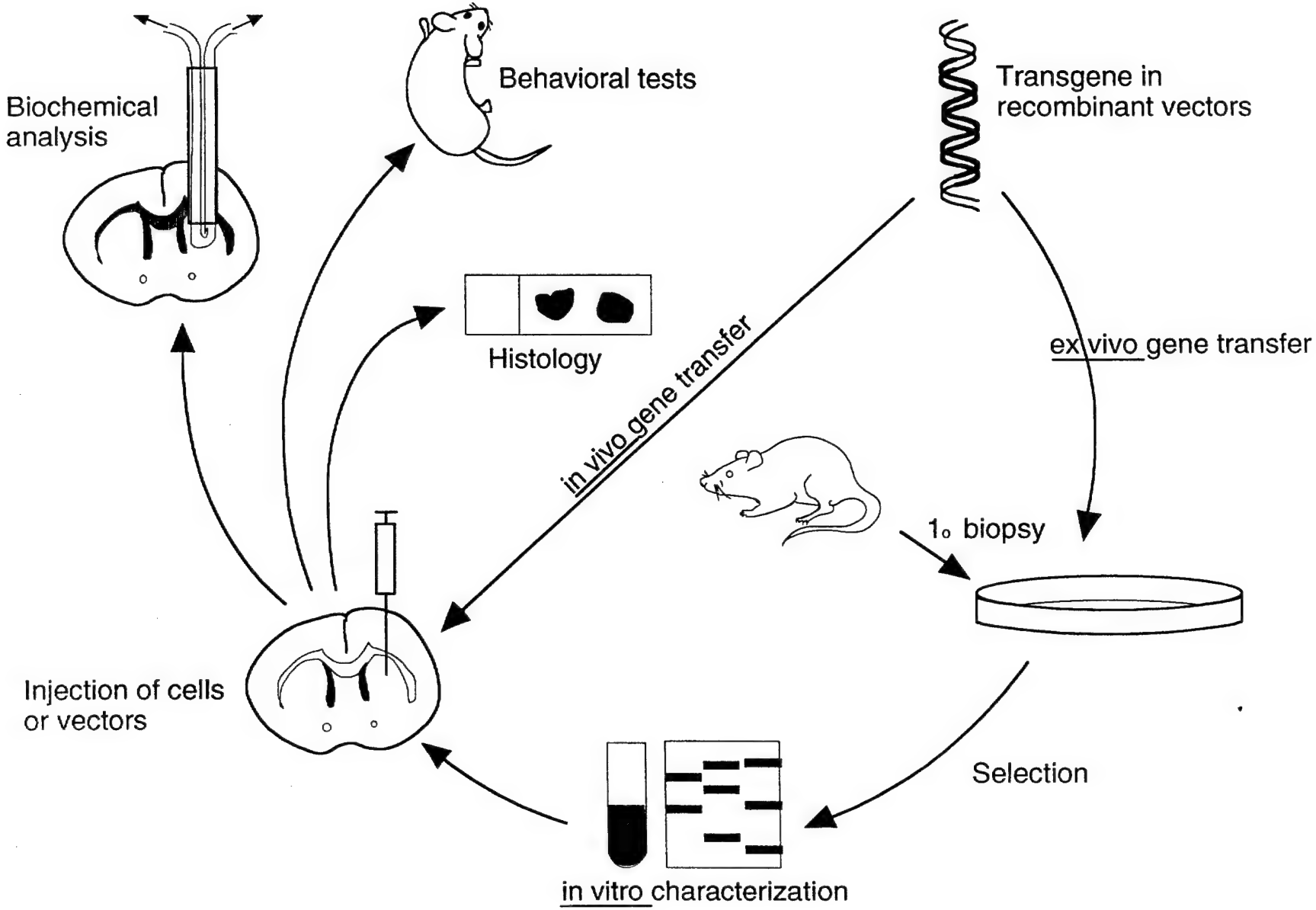
Figure Legend

Fig. 1 Experimental schemes for transferring genes into the CNS. Two general methods of gene transfer, *in vivo* and *ex vivo*, are schematically depicted. For details, please see the text.

Fig .2 An idealized scheme for general virus vectors. Wild-type viruses are engineered to produce virus plasmid vector which has minimal viral genes necessary for packaging the vector into the virus paricles. Other viral genes are deleted to reduce deleterious effect and make room for insertion of therapeutic genes. Viral components necessary to generate infective particles are introduced into the packaging cells separate from the virus plasmid vector. Packaging cells then produce infective virus particles containing the therapeutic transgene, but minimal or no virus genes.

Fig. 3 Dopamine replacement strategy for Parkinson's disease by gene transfer. A. Cells expressing tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1) are grafted into the striatum. The L-DOPA produced by these cells (dotted line) is converted by endogenous aromatic L-amino acid decarboxylase (AADC) to dopamine (solid line). B. Cells expressing AADC and vesicular monoamine transporter (VMAT) could be cografted with L-DOPA-producing cells to provide additional source of decarboxylation and storage of dopamine. C. An alternative strategy combines the use of AADC/VMAT cells with exogenous administration of L-DOPA. These cells increase the efficacy of L-DOPA by providing additional decarboxylation, and increased storage and sustained release of dopamine.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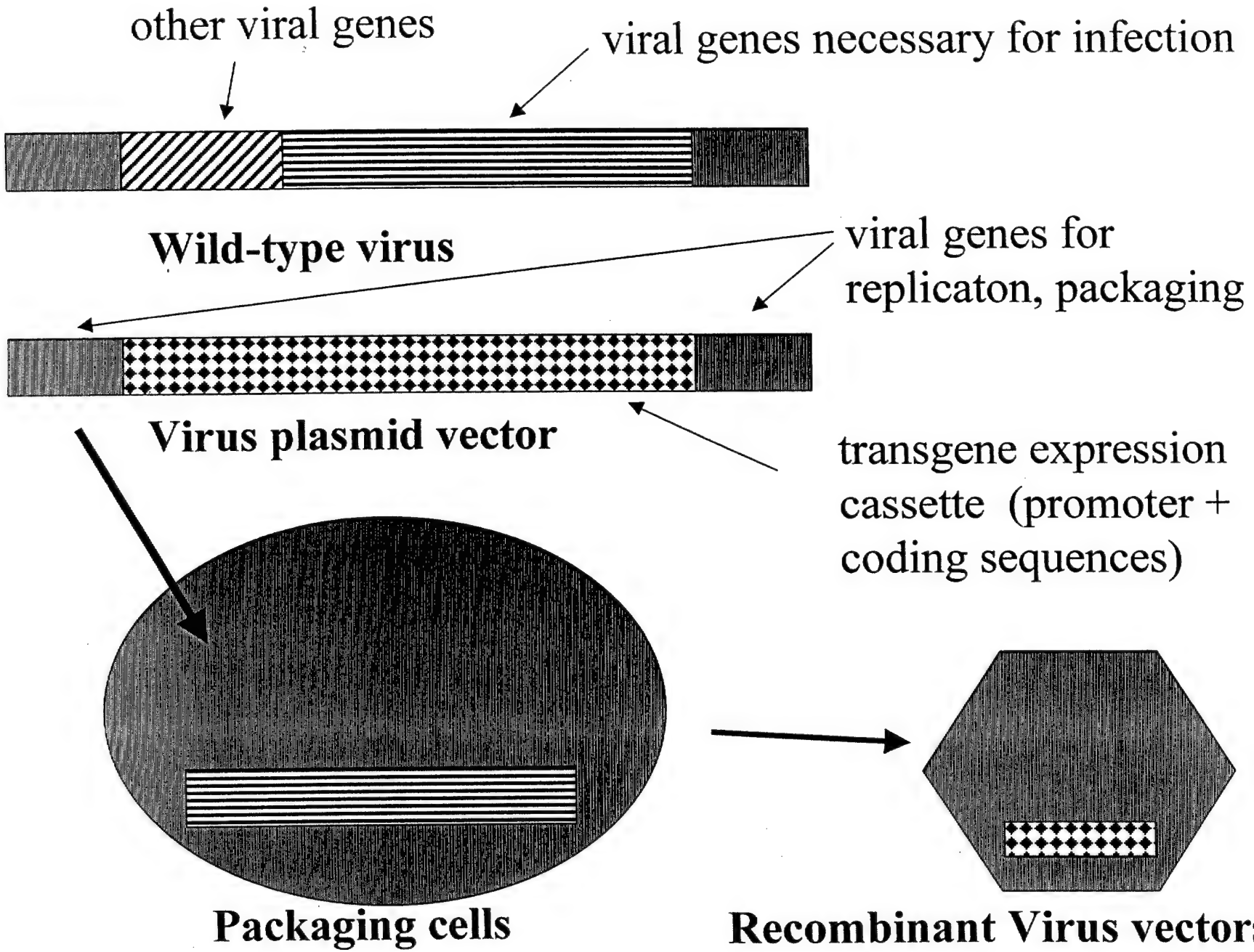
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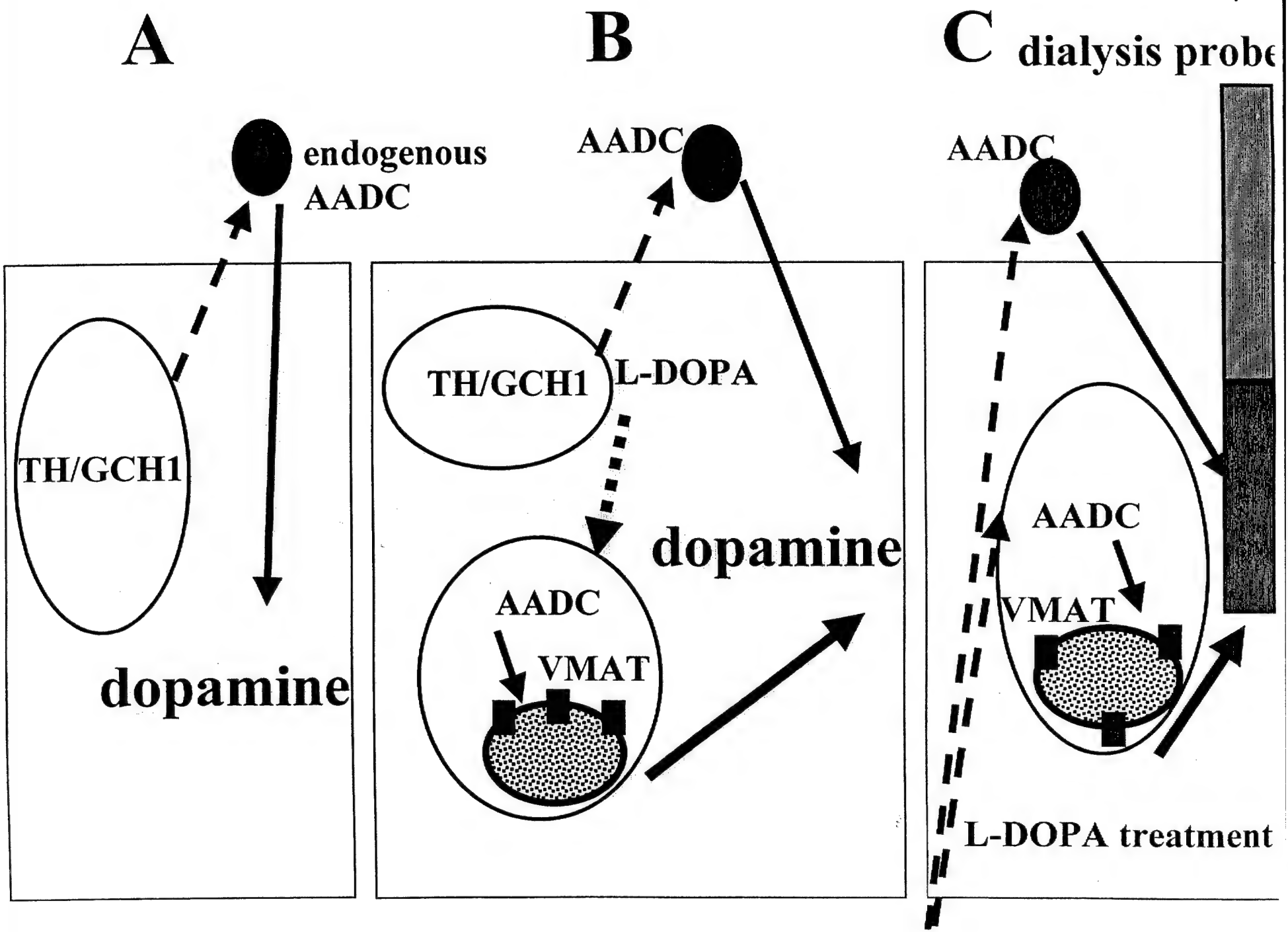
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Immunophilin Ligands and GDNF Enhance Neurite Branching or Elongation from Developing Dopamine Neurons in Culture

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Neurotrophic effects of immunophilin ligands have been shown in animal models of peripheral and central nervous system insult. To investigate the specific growth-promoting effects of these compounds, we examined the effects of various immunophilin ligands on primary dopamine (DA) neurons in culture and compared these with a well-known DA trophic factor, glial cell line-derived neurotrophic factor (GDNF). In neuronal cultures from Embryonic Day 14 ventral mesencephalon, enhanced *elongation* of DA neurites was observed with immunophilin ligands, which inhibited the phosphatase activity of calcineurin (FK506 and cyclosporin A) when compared to vehicle-treated cultures. This elongation was also observed with GDNF, known to exert its trophic effects through phosphorylation-dependent pathways. In contrast, immunophilin ligands that do not inhibit calcineurin (rapamycin and V-10,367) increased *branching* of DA neurites, suggesting that elongation is dependent upon maintained phosphorylation while branching is not. In addition, both V-10,367 and rapamycin antagonized the elongation effects of FK506 and induced branching. The antagonism of elongation (and reappearance of branching) illustrates the intrinsic abilities of developing DA neurons to *either* elongate or branch, but not both. We show that the immunophilin FKBP12 (12-kDa FK506-binding protein) is expressed in ventral mesencephalic neuronal cultures and colocalizes with DA neurons. This work elucidates the specific growth-promoting effects by which GDNF and immunophilin ligands modify developmental growth processes of DA neurons, via their interactions with intracellular targets.

Key Words: **immunophilin; FKBP12; neurite; GDNF; calcineurin; dopamine cultures; FK506; rapamycin.**

INTRODUCTION

The regulation of neurite elongation and branching is fundamental for establishing neuronal circuitry (49, 59). Recently, immunosuppressive drugs such as FK506 and cyclosporin A (CsA) have been shown to enhance neurite outgrowth from various cell lines and peripheral nerve preparations (50, 67), as well as primary CNS neurons in culture (10). These compounds also protect against peripheral and central nervous system insult (10, 26, 65, 70), alter long term potentiation, (LTP) and long term depresssion, (LTD) (21, 73), increase the rate of peripheral nerve regeneration (25), and regulate neurotransmitter release (58, 69). The observed effects are partially or fully mediated by specific intracellular pathways that are affected by these ligands, but that remain unclear in the context of neuronal cell systems. In the immune system, the immunophilin ligand FK506 complexes with the immunophilin FKBP12 (12-kDa FK506-binding protein). This drug–immunophilin complex then binds to and inhibits the phosphatase activity of calcineurin (48), augmenting the phosphorylation of several substrates (12, 28, 42, 52, 64, 68), which leads to inhibition of cytokine synthesis and immunosuppression (20). Cyclosporin A also inhibits calcineurin, but through its interaction with the immunophilin cyclophilin B.

High levels of FKBP12 and cyclophilin in the brain (68) and their colocalization with calcineurin suggested that the neuronal trophic effects may also act through this intracellular pathway. The neuroprotective effects seen with immunophilin ligands in models of neurodegenerative disorders have led to the design of small-molecule ligands that bind to immunophilins, but are not immunosuppressive (do not interact with calcineurin (3)). One of these novel immunophilin ligands, V-10,367, has an affinity to FKBP12 similar to that of FK506 (K_i of 0.5 nM) and does not decrease the phosphatase activity of calcineurin (3). However, trophic effects are retained even in the absence of calcineurin inhibition: V-10,367 potentiates neuronal growth factor (NGF)-induced neurite outgrowth from immortalized cells in culture (26). In addition, neuroprotective and regenerative effects of these novel immunophilin ligands have been obtained in animal models of peripheral and central nervous system insult (10, 26, 70). We

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TABLE 1

Immunophilin Ligands

Compound	Immunophilin interaction	Inhibition of calcineurin
FK506	FKBP12	Yes
CsA	Cyclophilin	Yes
Rapamycin	FKBP12	No
V-10,367	FKBP12	No

recently demonstrated neurotrophic effects of V-10,367 in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (10). Striatal dopaminergic (DA) innervation was spared from MPTP-induced degeneration in animals orally treated with V-10,367, while treatment with FK506 did not cause this effect. These studies suggest that inhibition of this phosphatase may not be required for the trophic effects obtained with these ligands in the nervous system.

To determine the specific effects of these compounds on the DA system, we evaluated the growth-promoting effects of these molecules on primary ventral mesencephalic (VM) DA neurons in culture. Comparisons were made between immunophilin ligands that interact with different intracellular targets and the trophic effects of glial cell line-derived neurotrophic factor (GDNF).

MATERIALS AND METHODS

Primary Ventral Mesencephalic Cultures

Primary cultures of DA neurons (previously shown to be 95% neuronal) were obtained from E14 Sprague–Dawley rat (Charles River, MA) ventral mesencephalon (VM) as described previously (10, 11). Briefly, tissue was dissociated by incubation in 0.025% trypsin solution (37°C, 15 min; Sigma) and triturated in a solution of DNAse (0.01%; Sigma) and trypsin inhibitor (0.05%; Sigma). Isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY) containing heat-inactivated horse serum (10%), glucose (6.0 mg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml; Sigma), and glutamine (2 mM; Gibco). Five hundred microliters of suspension containing 5×10^5 cells/ml was plated into each well of 24-well trays (Falcon), precoated with poly-L-lysine (Sigma), containing 500 μl of serum-containing (S+) medium. Unattached cells were aspirated after 1 h, and 1 ml of fresh S+ medium containing immunophilin ligands (Table 1), GDNF, or vehicle (DMSO diluted at equivalent concentrations (1:1000) per well) was added. At 1 day in culture, the medium was replaced with defined medium (containing N2 cocktail; Gibco) containing immunophilin ligands or GDNF (using 30 kDa as the molecular weight of GDNF, the doses of 0.001–100 ng/ml convert to 33 pM–3.3 nM). At 2 days *in vitro* (DIV), cultures were fixed for 1 h with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS).

Immunohistochemistry

Cells were incubated in primary antibody against tyrosine hydroxylase (TH; 1:500; Pel-Freez, Rogers, AK) for 48 h at 4°C; antibody binding was visualized with reaction in 0.05% 3,3′-diaminobenzidine (DAB, Sigma). To study the expression of the immunophilin FKBP12 within VM neurons, cultures were double-labeled with antibodies against TH and FKBP12 (1:2000; Pharmingen, CA) and processed for immunocytochemical detection with texas red- and fluorescein-conjugated secondary antibody (1:200; Jackson ImmunoResearch; West Grove, PA). Fluorescent images of double-labeled FKBP12 and TH was visualized using a confocal argon laser scanning microscope (Leika), processed with Leika Physiology Module Software.

Quantification of Neuron Survival and Neurite Outgrowth

For neuron survival, four random fields at each corner of each well were selected for cell counts, as previously described (10): at 200× magnification, and using an eyepiece grid, the number of cells in each 200 × 20-μm corner and center of the grid was counted (5 counts per field). Counts consisted of the total number of neurons in each grid region for neuronal viability (defined morphologically in phase contrast and confirmed by trypan blue exclusion test), as well as the number of TH+ neurons. For analysis of neurite elongation and branching, two fields from opposite sides of each well were captured in Adobe Photoshop, and 10 randomly chosen TH+ neurons per field were analyzed. Each neurite from a TH+ neuron was traced and measured (in millimeters) to determine *elongation* effects, and the number of neurites extending from a single TH+ neuron was counted to determine *branching* effects (Fig. 1). The measurements from the 10 TH+ neurons per field were averaged to obtain two measurements per well. Each dose of each compound was run in duplicate per experiment, and data are expressed as percentage control from two to four pooled experiments (each dose in duplicate per experiment, for $n = 8$–16 per dose). All comparisons were evaluated using analysis of variance (ANOVA) in JMP Version 3.1 (SAS Institute, NC). When significance was obtained, post-hoc Tukey–Kramer HSD was performed to compare significant differences between groups ($^*P < 0.05$). Error bars represent SEM.

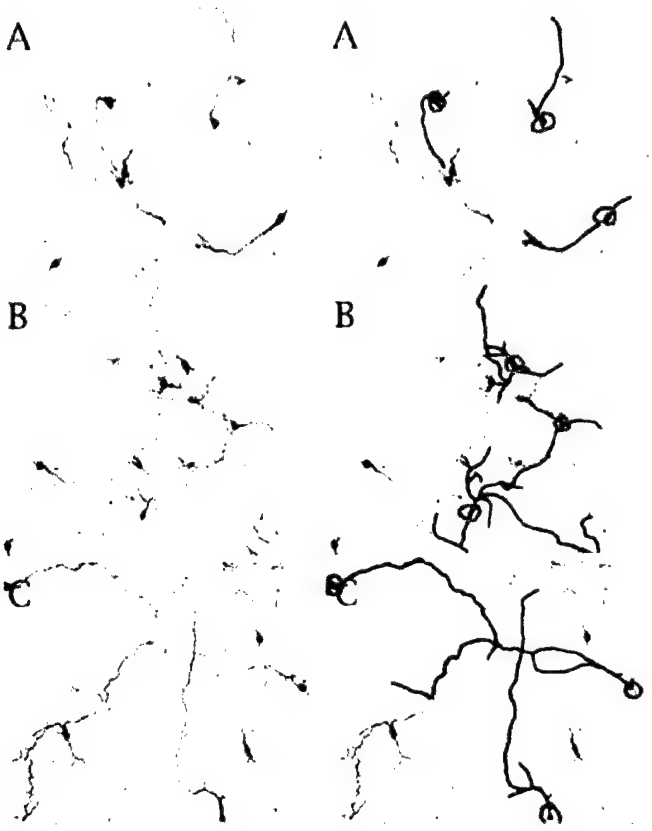

FIG. 1. Measurement of distinct effects of immunophilin ligands on neurite outgrowth of primary DA neurons. Parallel panels illustrate the method of measuring DA neurite outgrowth (stained for TH) in three representative cells (see Materials and Methods, and as quantified in Fig. 2): each TH+ neurite (outlined in red) was traced, counted, and measured from TH+ cell bodies (circled in green). (A) Neurite outgrowth of DA neurons from untreated primary cultures of E14 VM after 2 days in culture. (B) Enhanced *branching* of TH+ neurites from DA neurons after 2-day treatment with 1.0 μM V-10,367. (C) Enhanced *elongation* of TH+ neurites from DA neurons after 2-day treatment with 1.0 μM FK506.

RESULTS

To better define intracellular targets and mechanisms of the trophic effects observed with immunophilin ligands, we utilized a system of primary DA neurons from E14 VM. By immunostaining VM cultures for TH (used here as a marker for DA neurons, Fig. 1) we investigated the effects of immunophilin ligands (Table 1) on neurite outgrowth. After 2 days in culture, the vehicle-treated primary DA neurons developed fairly short, unbranched TH+ neurites (Fig. 1A). Treatment of cultures for 2 days with the immunophilin ligand V-10,367 produced a significantly larger number of neurites from each TH+ neuron (Fig. 1B), whereas treatment with FK506 increased the length of neurites from TH+ neurons (Fig. 1C). We quantified these effects as previously described (10). Briefly, each neurite from a TH+ neuron was traced and measured (in millimeters) to determine its length (elongation), and the number of neurites extending from each TH+ neuron was counted (branching). To determine effects of immunophilin ligands or GDNF on neuronal viability, the total numbers of surviving neurons were analyzed: quantification revealed no effect of treatment with any of the immunophilin ligands or GDNF on total number of neurons (mean number of neurons per field = 128.75; $P > 0.768$) or TH+ neurons (mean number of TH+ neurons per field = 8.31; $P > 0.258$) after 2 days in culture.

Elongation of Developing DA Neurites

To compare the trophic effects of immunophilin ligands to a well-known trophic factor for DA neurons, we first determined the specific effects of GDNF on neurite development. Neurite outgrowth was significantly influenced by 2-day treatment with GDNF (Fig. 2A): the average length of TH+ neurites was significantly longer than that of those in vehicle-treated cultures at a range of doses. We then compared this effect with two immunophilin ligands; FK506 (Fig. 2B) and CsA (Fig. 2C) that bind their respective immunophilins and subsequently inhibit calcineurin. Both FK506 and CsA significantly enhanced elongation of neurites from TH+ neurons. We then analyzed two immunophilin ligands that do not inhibit calcineurin: V-10,367 (Fig. 2D) and rapamycin (Fig. 2E). These compounds had no effect on the length of TH+ neurites. Interestingly, rapamycin showed a significant decrease in the length of neurites at some doses (Fig. 2E).

Branching of Developing DA Neurites

We next determined whether GDNF could affect the number of neurites extending from primary DA neurons. In contrast to elongation, neurite branching was not enhanced with GDNF (Fig. 3A). On the contrary, there tended to be fewer neurites per TH+ neuron than in vehicle-treated cultures at one dose. FK506 did not enhance branching at the doses that caused elongation of neurites (Fig. 3B); however, at one low dose (0.01 μM), a higher number of neurites was observed. The immunophilin ligand CsA had no effect on branching (Fig. 3C). In contrast, the two compounds that do not inhibit calcineurin (V-10,367 and rapamycin) showed significantly enhanced branching of TH+ neurites (Figs. 3D and 3E, respectively). The most robust effect with V-10,367 was observed at 0.05 μM, when DA neurons developed 2× the number of TH+ neurites that the vehicle-treated cultures developed.

Relationship between Branching and Elongation of Developing DA Neurites

In all cases where a compound produced a significant effect on DA neurite development (as previously ob-

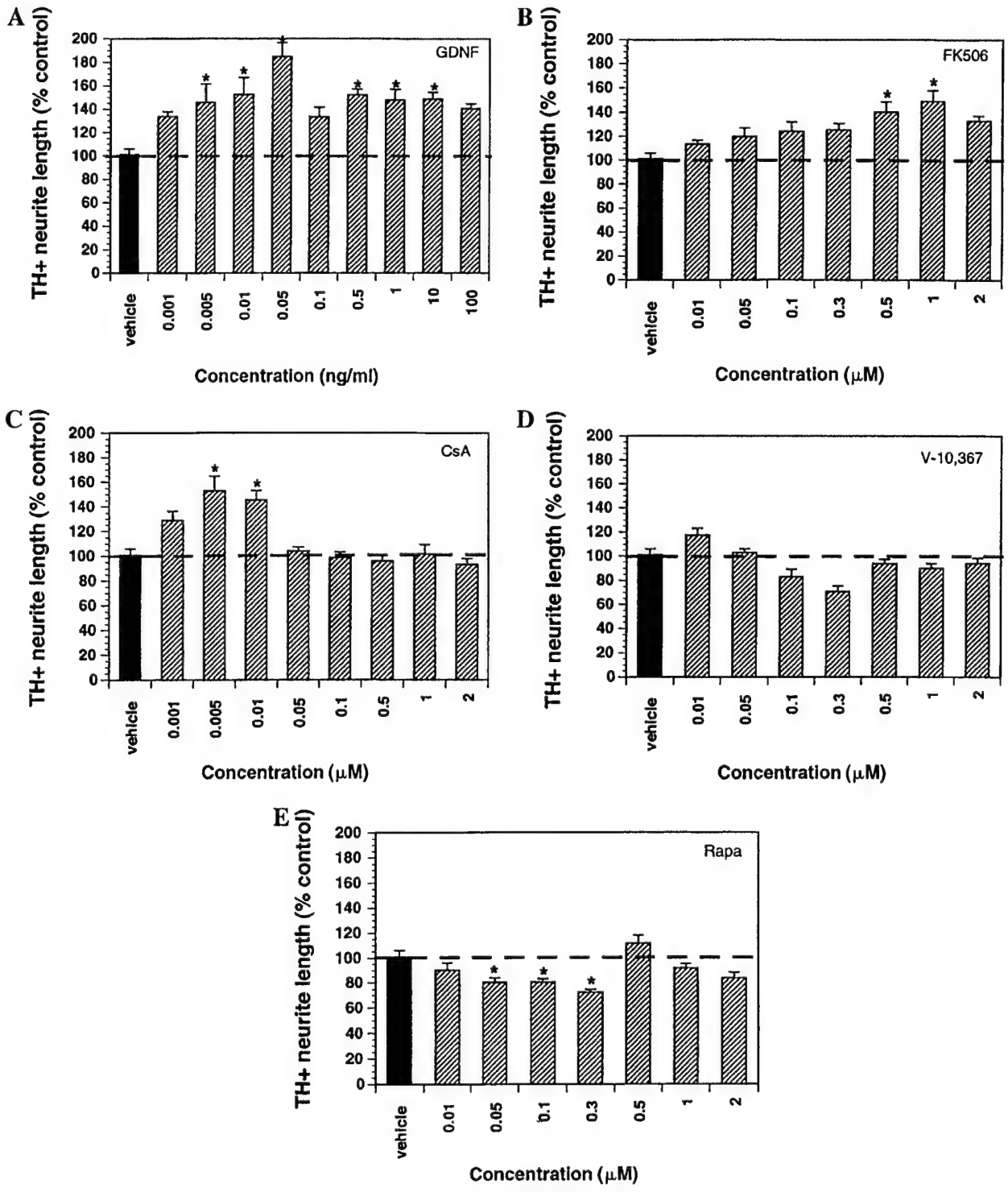

FIG. 2. Elongation of developing DA neurites. Length of TH+ neurites (percentage control) from primary cultures of E14 VM after 2 days of treatment. GDNF produced significantly longer neurites from TH+ neurons (A), as did FK506 (B) and CsA (C). The immunophilin ligand V-10,367, which does not inhibit the phosphatase calcineurin, showed no change in length of TH+ neurites (D), while rapamycin, which also does not inhibit calcineurin, showed a significant decrease in length of TH+ neurites (E). Tukey–Kramer HSD, $*P < 0.05$; error bars represent SEM.

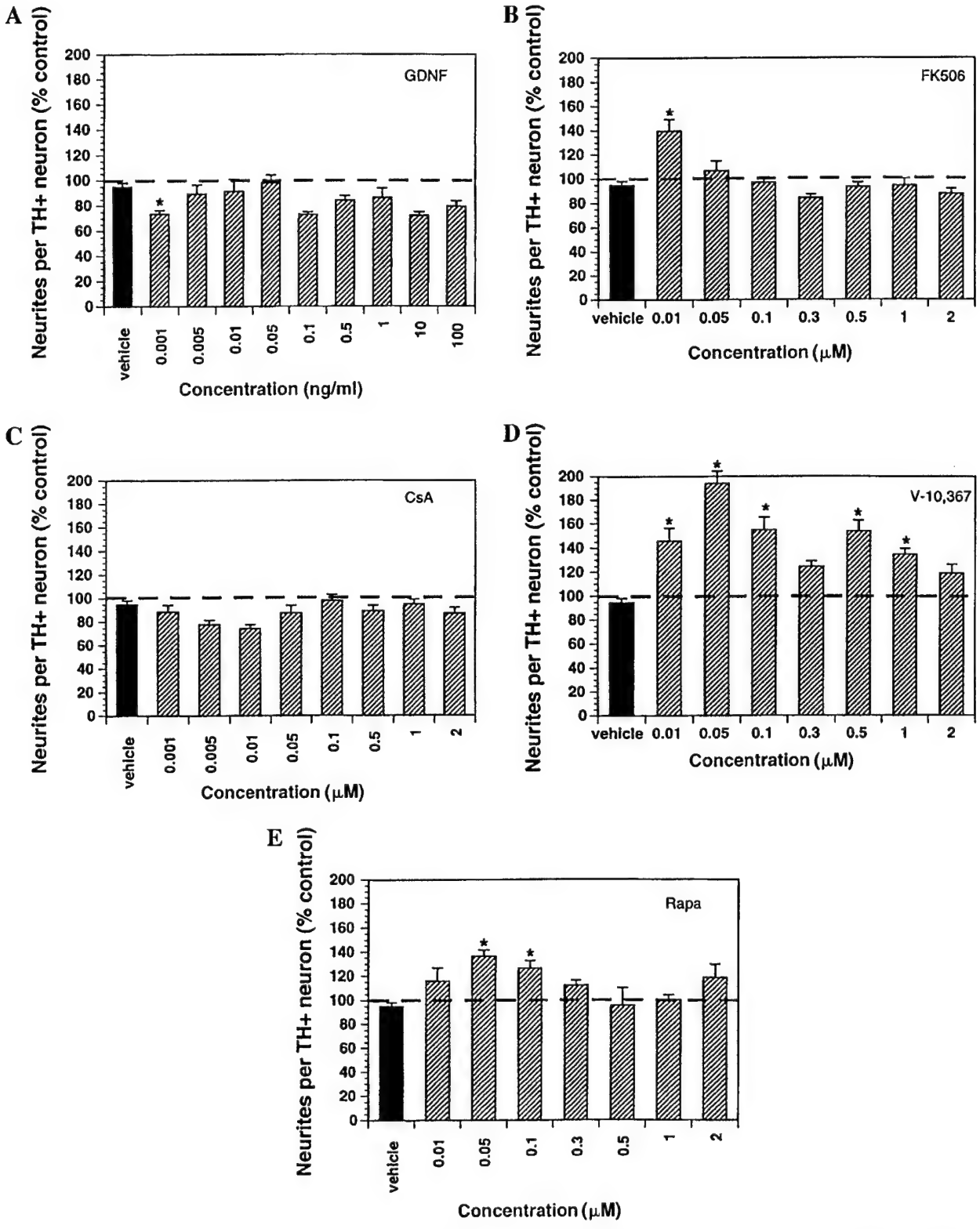

FIG. 3. Branching of developing DA neurites. Number of TH+ neurites (percentage control) from primary cultures of E14 VM after 2 days of treatment. GDNF showed a decrease in branching of TH+ neurites only at the lowest dose (A), while FK506 showed a slight branching effect at its lowest dose (B). CsA showed no effect on branching of TH+ neurites (C). V-10,367 (D) and rapamycin (E), compounds that do not inhibit the phosphatase calcineurin, both showed significantly enhanced branching of TH+ neurites. Tukey–Kramer HSD, $*P < 0.05$; error bars represent SEM.

served with classic neurotrophic factors (36)), a bell-shaped dose–response curve was apparent (see Figs. 2 and 3). In several cases when neurons showed enhanced elongation they did not simultaneously show increased branching, and vice versa. For instance, two doses of rapamycin (0.05 and 0.1 μM) significantly enhanced branching (Fig. 3E), whereas these doses showed significantly shorter TH+ neurites (Fig. 2E). Conversely the elongation produced by 0.005 and 0.01 μM CsA (Fig. 2C) was paralleled by a trend toward decreased branching at these doses (Fig. 3C). Similarly, the only dose of FK506 that did induce branching (0.01 μM, Fig. 3B) showed the lowest value of neurite length (Fig. 2B).

Pharmacological Targets of Trophic Effects

To further define the intracellular mechanisms involved in the elongation and branching observed, we determined the presence of the immunophilin FKBP12 in VM cultures. FKBP12 was expressed in these neurons after 2 days in culture (Fig. 4A) and colocalized with TH+ neurons (Figs. 4B and 4C). We used combinations of V-10,367 or rapamycin with FK506 to determine whether the elongation effect of FK506 involved signaling through calcineurin via its interaction with FKBP12. Rapamycin and V-10,367 also bind FKBP12 and compete with FK506 for FKBP12 binding sites. FK506 (1.0 μM) did not elongate TH+ neurites in the presence of V-10,367 or rapamycin (0.5 and 1.0 μM, Fig. 4D). This effect was observed with 0.5 μM FK506 as well (data not shown). However, V-10,367 and rapamycin were able to induce branching in the presence of FK506 (Fig. 4E).

DISCUSSION

Distinct Growth-Promoting Effects of Immunophilin Ligands

In the present study, we show that immunophilin ligands can enhance branching or elongation of neurite outgrowth from DA neurons. The identification of distinct aspects of neurite development allowed us to pharmacologically dissect specific effects of various immunophilin ligands and compare their effects with those of GDNF. The distinction between branching and elongation of neurites has previously been demonstrated when developing DA neurons are grown in the presence of growth factors (1, 5, 71) or their target striatal cells (14, 34, 62). For instance, exposure of VM neurons to conditioned medium from VM induces growth of dendrite-like neurites (short with a high number of branches), while striatal-conditioned medium stimulates growth of axon-like neurites (62). Though we did not differentiate between axons and dendrites (90% of neurites were MAP+, making selective analysis unreliable, Costantini *et al.*, unpublished observations), independent regulation of axonal and dendritic initiation and elongation from DA neurons may play a role in the effects observed here. Studies have shown GDNF-induced neurite outgrowth from peripheral (16, 44) and VM neurons in culture (18, 36, 47) or after transplantation of VM tissue into adult brain (2, 29, 40, 61, 63, 80). Though the most prominent influence of GDNF upon developing DA neurons is enhanced survival (17, 43, 46), we did not observe this effect, most likely due to the short time course of our studies.

Several studies have observed enhanced neurite outgrowth with immunophilin ligands that do not inhibit calcineurin (26, 67); however these studies utilized transformed cell lines (dependent on NGF for outgrowth) and DRG explants and did not quantify differences between elongation and branching. The primary DA neuron cultures used in the present study were chosen as a more physiologic system for analyzing neurite outgrowth than growth factor-responsive cell lines or peripheral culture systems.

Generally, growth-promoting effects of neurotrophic factors depend on receptor activation. The primary intracellular receptors for the immunophilin ligands are FKBPs and cyclophilins, immunophilins that are highly expressed in the adult rat brain (13, 68). The expression of FKBP12 in fetal VM cultures shown here suggests a physiological role for this protein in developing DA neurons. The observed expression of FKBP12 in all cells in VM cultures suggests that the immunophilin ligands may also affect the GABA neurons present in these cultures. However, these GABA neurons do not extend neurites to the same extent as DA neurons after 2 days in culture. GABA markers stained approximately 90% of the cells and neurites, making analysis of neurites from individual cells unreliable. No obvious changes in neurite outgrowth were observed in this neuronal population after treatment with immunophilin ligands or GDNF (Costantini *et al.*, unpublished observations). Alternatively, the immunophilin ligands may be acting through other immunophilins such as FKBP13 (77), FKBP25 (22), or FKBP52 (24, 72).

The receptor for CsA is the immunophilin cyclophilin (31), also expressed in the substantia nigra (13). Though CsA shows an affinity for its immunophilin that is 10-fold lower than FK506 has for FKBP12 (67), we observed enhanced elongation at lower doses with CsA than with FK506. This suggests that pathways other than the CsA/cyclophilin interaction may be involved. In contrast to our results, CsA prevented axonal elongation in cultured cerebellar neurons with no effect on neurite formation (19) and did not increase axonal regeneration in the sciatic nerve model (78), suggesting that cyclophilin-mediated trophic effects may not be active in these specific neuronal systems.

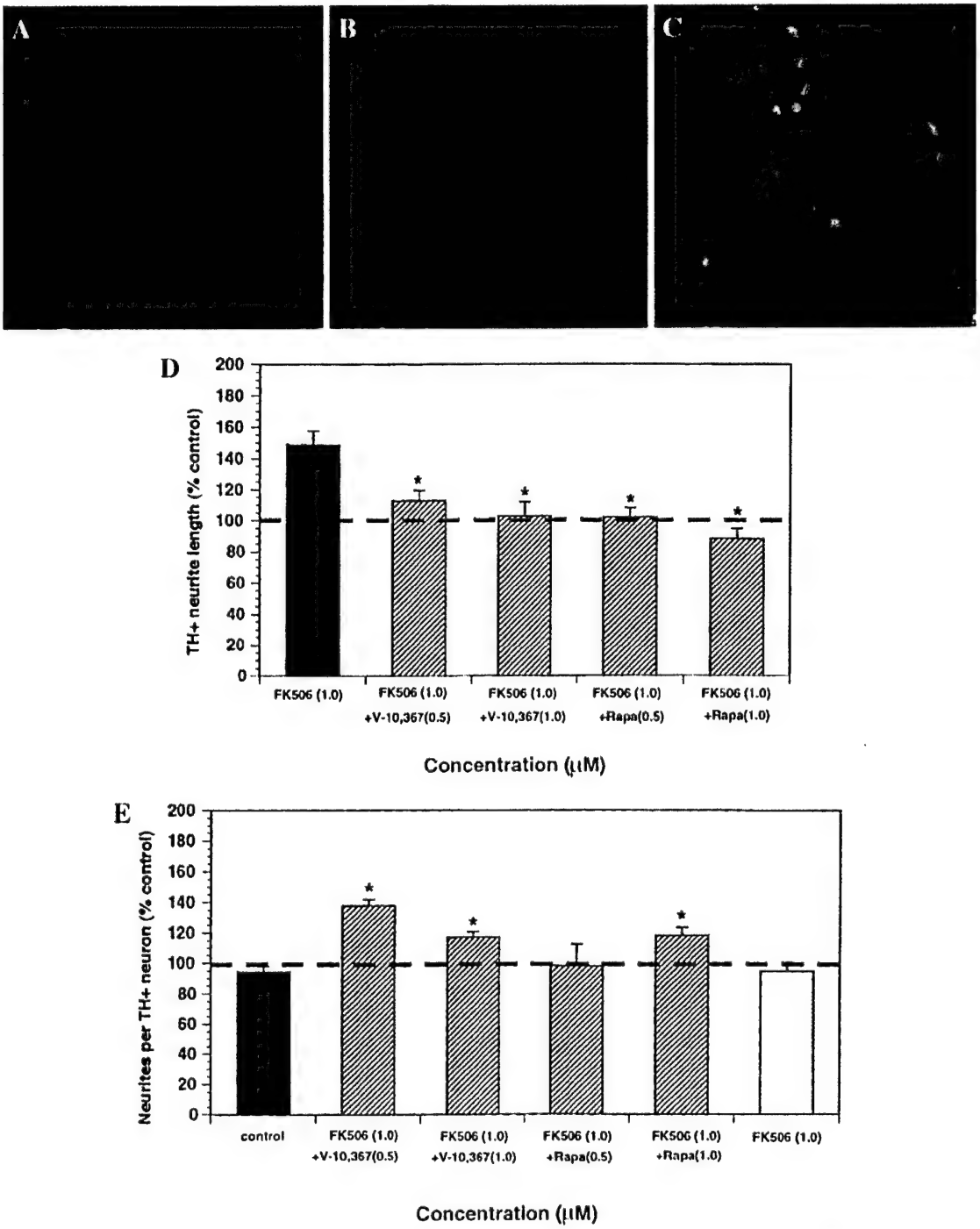

FIG. 4. Intracellular interactions of immunophilin ligands. (A) The immunophilin FKBP12 (green) is expressed in all neurons of primary cultures from E14 VM at 2 DIV. (B) The same field showing TH+ neurons (red). (C) Colocalization of FKBP12 and TH+ neurons (yellow). (D) FK506-induced elongation can be inhibited by compounds that compete for FKBP12-binding sites, such as V-10,367 and rapamycin. (E) Branching can occur with V-10,367 and rapamycin in the presence of FK506. Tukey–Kramer HSD, $*P < 0.05$; error bars represent SEM.

The bell-shaped immunophilin ligand dose–response curves in the present study suggest an optimal immunophilin ligand concentration for maximal trophic effects. Such effects have previously been explained by the "set-point hypothesis" of both growth factors and Ca+ levels (41, 55), where optimal trophic effects are observed with a mid level of growth factor or Ca+ level and low or high levels produce suboptimal or even toxic effects. Consistent with our present results (though inherent variability may also be responsible for the bell

shapes of these curves), a bell-shaped dose–response curve has been observed for optimal trophic effects of GDNF and FK506 (36, 78). Neurite sprouting and elongation are promoted only when intracellular Ca^{2+} levels are within a permissive range (53–55, 60). Immunophilin ligands have been shown to regulate Ca+ levels via their interactions with two Ca^{2+} channels found in the brain, the ryanodine receptor (RyR), and the inositol triphosphate receptor (IP3R) (7, 8, 37, 38, 66). Furthermore, these receptors are expressed in the primary VM neurons used in the present studies (Costantini, unpublished observations).

Role of Phosphorylation in Elongation or Branching of DA Neurites

We observed enhanced elongation with immunophilin ligands that inhibit the phosphatase activity of calcineurin (48) through their binding to their respective immunophilins (31, 32), as well as with GDNF which requires phosphorylation for its trophic effects (35). Calcineurin is expressed in the substantia nigra (13, 27), and its inhibition maintains the phosphorylation levels of several substrates (12, 28, 42, 52, 64, 68) that are critically involved in neuronal outgrowth (51, 54). Moreover, inhibition of calcineurin by a FK506 analog (ascomycin) stabilized F-actin in cultured hippocampal neurons (30), suggesting a role for calcineurin in cytoskeletal systems during neurite elongation (28).

In our study, the elongation observed with ligands inhibiting calcineurin was also observed with GDNF. The GDNF receptor system consists of GDNF family receptor α1 (GFR α1) and the tyrosine kinase Ret (39), both of which are expressed in developing VM (4, 18, 74, 79). Activation of Ret tyrosine kinase activity by GDNF results in formation of neuritogenesis-associated lamellipodia (75), and tyrosine kinase inhibitors suppress GDNF-induced neurite outgrowth in cell lines (35). Thus, the compounds in our study that showed enhanced elongation of DA neurites are known to maintain phosphorylation levels within the cell, either through kinase activation (GDNF) or through inhibition of calcineurin (FK506 and CsA). Assays determining phosphorylation states of growth-associated proteins, such as GAP43 (6), microtubule-associated proteins (45), nitric oxide synthase (12), actin depolymerization factor (56), and GTPases (23) are required to fully evaluate this hypothesis. Alternatively, the folding of cytoskeletal proteins, receptors, or ion channels (33) through the rotamase-inhibitory effects of immunophilin ligands may alter extension and branching of developing neurites (32, 33).

Given the enhanced branching of DA neurites observed with rapamycin and V-10,367, a mechanism independent of calcineurin inhibition for trophic effects must be in play. Other evidence supporting a calcineurin-independent trophic mechanism is the neuroprotection and regeneration observed with immunophilin ligands that do not inhibit calcineurin (10, 26, 67). We investigated the role of calcineurin in these growth-promoting effects by combining either V-10,367 or rapamycin with FK506. Rapamycin is a competitive inhibitor of FK506 due to its higher affinity for FKBP12 (15) (and we found that rapamycin produced its trophic effects on DA neurites at concentrations lower than those of FK506, reflecting a higher potency). Both rapamycin and V-10,367 antagonized the effects of FK506 on elongation, presumably by competing for FKBP12-binding sites and decreasing the ability of FK506 to inhibit calcineurin. At the lower doses of competing V-10,367 and rapamycin, FK506-induced elongation was blocked but no branching occurred. However, at the higher doses of V-10,367 and rapamycin, branching did occur, even in the presence of FK506. These results support the requirement for maintained phosphorylation to obtain enhanced elongation of DA neurites.

The antagonism of elongation (and reappearance of branching) elucidate the intrinsic abilities of developing DA neurons to *either* elongate or branch, but not both. During development, when DA axons reach their target, they stop elongating and begin to branch extensively (57). Growth cone motility is directly related to neurite branching and stabilizes outgrowth by holding elongation rate at a submaximal level (55). In this way, under conditions of neurite branching (as with V-10,367 and rapamycin), neurites would show reduced elongation.

In contrast to our observations, the enhancement of NGF-induced outgrowth from PC12 cells and DRG by FK506 was not blocked by rapamycin, yet rapamycin alone enhanced outgrowth in these systems (50, 70). In a separate study, FK506 and CsA *inhibited* the NGF-induced neurite extension and neuritogenesis from DRG, while rapamycin inhibited only extension (9). The intracellular pathways present in PC12 and DRG cells are different than those in the primary DA culture system used in the present study, in which we observe rapamycin-associated pathways contributing to increased branching of DA neurites.

In conclusion, this study provides insight into the growth-promoting effects of immunophilin ligands on the DA system. Dopaminergic neurons of the substantia nigra start elongating axons toward their striatal target soon after final differentiation around Embryonic Day 12; then these axons branch extensively to innervate the striatum (76). During this phase, the developing neuron (both in culture and in brain) is expressing and responding to specific proteins that affect neuritogenesis and elongation (6, 17). The contrasting effects we observed on branching and elongation by different immunophilin ligands and GDNF suggest that these molecules differentially modify the

activities, expression, or structural states of proteins involved in neurite outgrowth. These observations may be relevant to recent trophic effects observed *in vivo* with immunophilin ligands (10, 26, 67). Additional studies are required to further elucidate the biochemical and molecular pathways involved in these growth-promoting effects.

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ENHANCED AXONAL GROWTH FROM HUBCL-2 TRANSGENIC MOUSE DOPAMINE NEURONS TRANSPLANTED TO THE ADULT RAT STRIATUM

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ABSTRACT

Embryonic neurons transplanted to the adult CNS extend axons only for a developmentally defined time. There are certain intercellular factors that control the axonal extension, one of which may be the expression of the bcl-2 protein. We transplanted mouse fetal ventral mesencephalon cells overexpressing human bcl-2 and compared those grafts with wild-type F1 generation fetal tissue into rat hosts with complete dopamine fiber denervation. Rats were treated with cyclosporine to prevent rejection and the surviving grafts were analyzed for cell survival and outgrowth of dopaminergic fibers. The results demonstrate that the bcl-2 overexpression did not enhance cell survival in the striatal location. However, the bcl-2 overexpressing grafts maintained axonal growth and had a higher number of dopaminergic fibers at longer distances. These results confirm that overexpression of bcl-2 can maintain longer distance axonal growth in dopaminergic neurons and that genetic modification of embryonic donor cells may enhance their ability to reinnervate a neuronal target territory.

INTRODUCTION

The adult brain is less permissive for axonal growth and regeneration than the fetal or neonatal period. However, fetal axons show less restriction than adult axons in regrowth in adult brain environments. Typically, fetal dopaminergic (DA) cells transplanted to the adult striatum will grow and create a new zone of DA axons and synapses associated with behavioral recovery(Dunnett and Björklund, 1994). Careful histological analysis has shown that even with a large number of surviving DA neurons, only a part of the target immediately surrounding the graft is fully reinnervated with current transplantation methods. In contrast, during the host's neonatal period, implanted

fetal neurons migrate into the surrounding brain and extend axons for longer distances(Isacson and Deacon, 1997; Loschinger et al., 1997; Nikkah et al., 1995). The host age at which transplanted embryonic VM neurons show much restricted axonal elongation occurs between postnatal day 8 and 20(Isacson and Deacon 1997; Holm and Isacson 1999).

Obviously, a number of factors and redundant molecules will control axonal growth. Key factors include growth-associated proteins (GAPS), activated cytoskeletal proteins, calcium, cyclic AMP and other second-messenger systems(Igarashi et al., 1995; Lnenicka et al., 1998; Loschinger et al., 1997; Nikolic et al., 1998; Wang and Zheng, 1998). The bcl-2 molecule has been proposed as relevant for inducing and maintaining axonal growth(Chen et al., 1997; Holm and Isacson, 1999). The bcl-2 gene was discovered as a proto-oncogene of a follicular lymphoma translocation t (14;18). In non-pathological roles, bcl-2 is considered an anti-apoptotic factor against ischemia(Martinou et al., 1994a; Martinou et al., 1994b), traumatic brain injury(Clark et al., 1997), growth factor deprivation, or generation of free radicals(Merry and Korsmeyer, 1997).

Bcl-2 is highly expressed in the brain in the middle to late gestational phase. These levels are reduced after birth, except in regions with late differentiation, for example, the dentate gyrus(Bernier and Parent, 1998a; Bernier and Parent, 1998b; Merry et al., 1994) (Merry et al., 1994; Bernier and Parent, 1998)(Bernier and Parent, 1998b; Merry et al., 1994). The highest levels of bcl-2 occur during the fetal phase and are seen in the cells of the ventricular zones and in post mitotic neurons during the period of naturally occurring cell death. Bcl-2 expression is high in the cortical plate, peaking around E16. Bcl-2 levels decline in most CNS neurons over the adult period, but remain relatively higher in neurons of the PNS.

Naturally occurring cell death (NOCD) is a prominent feature of the developing CNS. It can involve as much as 80% of the neurons, and such histogenetic cell death may

be interpreted as a competition for growth factors(Oppenheim, 1991). The period of bcl-2 expression in the developing nervous system matches the phases of NOCD with the axons at their target zones(Martinou et al. 1994a; Martinou et al. 1994b). This suggests an involvement in selective sparing of neurons during this period. Mice overexpressing bcl-2 show reduced neuronal loss during NOCD(Martinou et al. 1994a; Martinou et al. 1994b).

The peaks of bcl-2 expression coincide with axonal elongation(Merry et al., 1994), suggesting a protection function distinct from its anti-apoptotic actions. Chen et al. (Chen et al., 1997) used mice overexpressing human bcl-2, coupled to a neuron specific enolase promoter. Retino-tectal co-cultures prepared from these mice maintained axonal growth to the tectum at E18, in contrast to wild-type control cultures(Chen et al., 1997; Holm and Isacson, 1999). Reduced levels of bcl-2 decrease axonal growth ability of sensory neurons(Hilton et al., 1997) and in co-cultures prepared from E16 *knockouts* for bcl-2 of retina and tectum, homozygote cultures showed an 80% decrease axonal growth to the tectum compared to wild type cases, E18, the time when axonal extension in this system normally stops. Immunohistochemistry of fetal retinal tissue also indicates an absence of bcl-2 immunoreactivity in normal retinas at E18(Chen et al., 1997). The ability of the PNS to regenerate axons and the persistence of bcl-2 in these cells are consistent with the idea of a growth promoting effect of bcl-2. Notably, bcl-2 expression has been described in the superior cervical ganglion and the dorsal root ganglion in mice at 5 months(Merry et al., 1994). Furthermore, axotomized retinal axons in P4 bcl-2 overexpressing mice managed to grow across a knife cut lesion to innervate the tectum(Chen et al., 1997). A role of bcl-2 in axonal growth is also indicated by the work of Oh and colleagues (Oh et al., 1996) in a cell line showing that vector expressed human bcl-2 causes increased neurite formation. Overexpressing bcl-2 in a neural crest derived

cell line by adding sense cDNA results in enhanced axonal outgrowth(Zhang et al., 1996).

METHODS

Cell culture

E13 mice were obtained by backcrossing human Bcl 2 (hubcl-2) overexpressing hemizygote males (line 73, D. F. Chen, MIT, MA) with wild-type F1 generation female progeny and primary ventral mesencephalic neuronal cultures (95% neuronal) were obtained, as previously described(Costantini et al., 1998; Costantini and Snyder-Keller, 1997). Briefly, tissue was dissociated by incubation in 0.025% trypsin solution (37°C, 15 min; Sigma, St. Louis, MO) and triturated in a solution of DNAse (0.01%; Sigma) and trypsin inhibitor (0.05%; Sigma). Isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) containing heat-inactivated horse serum (10%), glucose (6.0mg/ml), penicilin (10,000U/ml), streptomysin (10mg/ml; Sigma), and glutamine (2mM;GIBCO). Five hundred microliters of suspension containing 1 x 10^6 cells/ml were plated onto glass coverslips precoated with poly-L-lysine (Sigma) in each well of 24-well trays (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) containing 500 μl of serum-containing (S+) medium. Unattached cells were aspirated after 1 hr, and 1 ml of fresh S+ medium was added. After 1 day of culture, the medium was replaced with defined medium (N2 cocktail GIBCO). After two days, cultures were fixed for 1 hr with 4% paraformaldehyde/4% sucrose in PBS. Fixed cells were preincubated in 10% normal goat serum (NGS), then incubated in polyclonal antibody against tyrosine hydroxylase (TH, Pel-Freeze, AK; 1:500) and monoclonal antibody against human bcl-2 (Santa Cruz, CA; 1:100) for 48 hours at 4°C. After additional rinsing 3x10 min. in PBS, cultures were incubated for 1 hour in PBS with 2% NGS, biotinylated goat anti-mouse (Sigma, St. Louis, MO; 1:200) and biotinylated goat

anti-rabbit (Vector Labs, Burlingame, CA; 1:200) secondary antibodies at room temperature. After rinsing in PBS, cultures were subsequently incubated in avidin-biotin complex with peroxidase (Vectastain ABC kit ELITE; Vector Laboratories, Burlingame, CA). Antibody binding was visualized by reaction in 0.05% 3,3-diaminobenzidine (DAB; Sigma) with 0.04% H_2O_2 for 3 min. Controls with omission of primary antibody were performed to verify the specificity of staining.

6-OHDA lesions

Adult female Sprague Dawley rats (300 g, Charles River Laboratories) received unilateral 6-OHDA (4μl of 3 μg/μl) by stereotaxic injection into the medial forebrain bundle using a 10μl Hamilton syringe at the following coordinates: AP: -4.4, L: -1.4 and V: -7.8, incisor bar: -2.3mm. 6-OHDA was infused at a rate of 1 μl/min, and the needle was left in place for an additional 2 min prior to withdrawal. To verify a complete DA denervation, one-to-two weeks post-lesion, animals were tested for amphetamine-induced (5mg/kg) rotational asymmetry using automated rotometers (San Diego Instruments)(Ungerstedt and Arbuthnott, 1970).

Ten rats with a net ipsilateral rotation greater than 800 rotations per 90 min (reflecting an approximate 97% DA depletion) were divided into two experimental groups balanced with respect to pre-transplant rotation scores. The end-point for this study was histological.

Transplantation of VM cells

Ventral mesencephalons (VM) of E13-E14 fetuses from hubcl-2 transgenic (line 73, D. F. Chen, MIT, MA)(Martinou et al., 1994a; Martinou et al., 1994b) or wild type C57BL/6J mice were dissected. The dissected pieces from each litter were pooled, incubated in 0.1% trypsin for 20 min., washed with Ca/Mg free HBSS containing 0.1%

DNAse and triturated in the same solution through a series of fire polished pipettes of diminishing diameters, until a milky suspension with scattered cell clumps was achieved. Cell counts were carried out using acridine orange/ethidium bromide labeling to determine cell concentration. The suspension was diluted to a concentration of 100,000 cells/μl.

Two μl of the cell suspension was injected into the host denervated striatum using a 10μl Hamilton syringe and a 45° beveled needle, inner diameter 0.26mm and outer diameter 0.46mm at a rate of 0.5μl/min followed by a 2-min pause before retracting the needle (coordinates relative to bregma: AP:+1.0, L: -2.5, V:-5.0, 4.0, incisor bar: -3.3). One group (n=5) received cells from wild type mice and the other group (n=5) was implanted with cells from bcl-2 transgenic mice. One day prior to transplantation procedure, animals were given 20 mg/kg of the immunosuppressant cyclosporin A (CsA, Sandimmune, diluted in olive oil, sc; Sandoz, East Hanover, NJ). This treatment continued at a lower dose (10mg/kg/day, s.c) until the animals were sacrificed.

Histological fixation and immunostaining of brain sections

Five weeks post-transplantation, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, ip) and perfused intracardially with 100ml heparinized saline followed by 200ml 4% paraformaldehyde (PFA) in 0.1M PBS, pH ~7.4. The brains were post-fixed for 6-8 hours in the same fixative before being transferred to 20% sucrose in 0.1M PBS for cryoprotection. Following equilibration, series of 40μm coronal sections were cut on a freezing microtome and collected in PBS.

Brain sections were immunostained with antibodies to tyrosine hydroxylase (Pel-Freez, AK;1:500 and hubcl-2 (Santa Cruz, CA;1:100) (All immunohistochemistry was performed on free floating sections, using a standard avidin-biotin technique. The sections were incubated in a 0.3% hydrogen peroxide solution containing 50% methanol

in PBS (pH 7.4) for 20 min to eliminate endogenous peroxidase. After rinsing three times in PBS, the tissue was incubated in 10% normal goat serum (NGS) dissolved in PBS containing 1% bovine serum albumin, 1% NGS, and 0.1% Triton X-100. The following day, sections were rinsed in PBS, then put through two washes of 5% NGS (10 min each) before incubation in secondary antibody solution (biotinylated goat anti-rabbit Vector Labs, Burlingame, CA or biotinylated goat anti-mouse Sigma, St. Louis, MO; 1:200 with 2% NGS in PBS) After being rinsed in PBS, the sections were incubated in avidin-biotin complex (Elite ABC kit, Vector Labs) for 90 min. After one wash in PBS, the tissue was transferred to Tris-buffered saline (TBS, 0.05M, pH 7.8) before being developed in 3,3'-diaminobenzidine tetrahydrochloride dissolved in TBS, with 0.03% hydrogen peroxide added. The sections were rinsed in TBS, then PBS before being plated on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), dehydrated through a series of graded ethanols and xylenes, then coverslipped with Permount (Fisher Scientific).

Determination of cell survival and outgrowth from grafted DA neurons

Surviving TH cells were counted over the extent of the grafts with Abercrombie corrections (ref). Since all grafts in the adult brain survived as isolated aggregates and were located in different striatal regions due to surgical variation, we established a semi-quantitative measure of fiber outgrowth for such grafts. For each rat, sections from AP +1.6 to AP –1.4, where the center of the grafts was observable were digitally captured at low power magnification on a Zeiss Axioplan microscope and imported into Adobe Photoshop 5.0 software. The graft was outlined and concentric circles (Fig. 1; L1, L2, L3) at fixed distances were drawn from the center of the graft going to the periphery (Fig. 1) and the number of TH positive cells were counted. We also measured the distance from the edge of the grafts to the circles to control for variance in graft size. As shown in Figure 1, the counting grid was divided into 8 sectors. The number of fiber in each sector

crossing the concentric circles was counted. The distance from the center of the graft to the edge of the graft (graft area), and to L1, L2 and L3 were measured. All measurements were done using an image analysis system based in Image 1.41 (NIH, Bethesda, MD).

Statistical analysis

The statistical analysis was carried out using the JMP program (version 3.1.6; SAS Institute, Cary, NC). Differences between groups were considered significant when $p<0.05$. One-way ANOVA was used to compare cell survival between groups. A two-way ANOVA with correction for multiple comparisons was performed to assess differences in axonal outgrowth between groups.

RESULTS

Expression of human bcl-2 (hubcl-2) in fetal ventral mesencephalic dopamine neurons in vivo *and* in vitro

To confirm the hubcl-2 expression in the adult substantia nigra of the transgenic mice, we stained the post-mortem tissue of 2 month old genotyped heterozygous mice. The hubcl-2 was highly expressed in neurons of the substantia nigra compacta (SNc) and the ventral tegmental area (VTA) (Fig. 2a) whereas variable and lower levels of expression were observed in to other brain regions (data not shown). To address expression and survival of dissociated VM, we performed *in vitro* studies. We found that in E-13 hubcl-2 cultured ventral mesencephalic neurons, approximately 50% of the neurons co-expressed TH and hubcl-2 (Fig. 2b). This proportion of co-expression was expected since the cultures were prepared from tissue dissected and pooled from the same litter, which according to Mendelian genetics would consist of 50% transgenic and 50% wild-type embryos.

Five weeks following transplantation of dissociated embryonic E13 VM into the rat striatum, the ratio of hubcl-2 and non hubcl-2 expressing neurons (50/50) (Fig. 2c) indicating that overexpression of hubcl-2 does not increase the survival of these neurons. Generally, the surviving grafts were small in both transplanted groups (0.5-1.0 mm3) but all contained high proportion of Th positive neurons (Fig.1 and Fig. 2d). The implanted neurons innervated the completely denervated host striatum (Fig. 2d). Axons could be followed from the center of the graft aggregates into the striatal neuropil.

Fiber outgrowth from wild type and hubcl-2 overexpressing dopaminergic neurons grafted to adult striatum

When the total number of TH-positive neurons within the hubcl-2 grafts was compared with wild type controls, no significant difference was detected ($p>0.05$) (Fig. 3a). To determine the extent of axonal outgrowth from the grafted neurons, we performed image analysis and semi-quantitative measurements of TH-positive fiber outgrowth from the center of the graft (Fig. 1) (See methods for details). The number of TH-positive fibers crossing 3 circles at set distances from the center of the graft was counted and compared between grafts containing hubcl-2 overexpressing neurons or grafts with only wild type neurons. The control for graft shape and size, the distance of the graft's edge in a counted sector was determined while a tendency for smaller graft were seen in hubcl-2 containing graft, the difference did not reach significance ($p>0.05$). The total number of TH-positive fibers crossing the innermost circle (L1) was not different between hubcl-2 and control grafts (Table 1 and Fig. 3b). Similar results were obtained from TH fibers counts of crossings at the middle circle (L2) (Table 1 and Fig. 3b) but there was a significant increase in the number of fibers from hubcl-2 grafts crossing the outermost circle (L3) compared to control grafts. Statistical analysis (two-way ANOVA; $p<0.05$) revealed that only control grafts had a significantly reduced number of crossings over

distance. In contrast, hubcl-2 grafts grew continuously throughout the distances defined by the concentric circles (Fig. 3b).

DISCUSSION

In this study, we have grafted VM neurons from mice overexpressing hubcl-2(Martinou et al., 1994a; Martinou et al., 1994b) into the striatum of immunosuppressed 6-OHDA lesioned rats. TH-positive cell survival was not significantly different between hubcl-2 grafts and controls, supporting previous observations(Schierle et al., 1999). Fibers of grafts composed of hubcl-2 expressing neurons grew for significantly longer distances into the neuropil compared to controls.

Enhanced axonal growth in hubcl-2 expressing mesencephalic grafts

The grafts of fetal DA neurons always aggregate in SN-like small structures in the adult host. Since the range of placements is always variable (1-2 mm AV/LM) the most appropriate method for axonal counts is concentric to the actual graft. Since all rats were fully lesioned and grafts outlined by hubcl-2 and TH cell body, the TH fiber staining in a radial appearance of fibers (from the graft center) allowed for fiber counts between groups of animals(Costantini and Isacson, 2000). The immunohistochemical detection of hubcl-2 was restricted to the cell bodies of the grafted neurons, thus limiting the possibility to trace individual axons from hubcl-2 expressing neurons. An alternative suggestion to the increased fiber density surrounding the hubcl-2 grafts aggregates would be a regeneration of host DA fibers. This is highly unlikely given that the rat hosts were selected for transplantation on the basis of drug-induced measures for complete denervation, which was confirmed in all post-mortem studies. The typical concentric outgrowth of DA fiber from the transplants characteristically forms a halo on

the background of a denervated host striatum. Consequently, no fibers were seen in the interface between grafts derived DA fibers and denervated host striatum. This interpretation is also supported by work by Schierle et al.(Schierle et al., 1999) where two grafts of hubcl-2 overexpressing fetal VM grafts were placed into the denervated rat striatum and densitometry measurements indicated increased volume of TH-innervation compared to wild-type control grafts.

Ability of embryonic neurons to extend axons in the adult brain

In normal development, axonal growth occurs in spurts interrupted by growth cone collapse(Igarashi et al., 1995; Keynes and Cook, 1995; Loschinger et al., 1997). It appears that molecular gradients of attractive and inhibitory cues provide axonal orientation by inducing asymmetric growth cone collapse and extension(Keynes and Cook, 1995). Axons from transplanted fetal neurons can grow extensively in neonatal, but not in adult host brain(Isacson and Deacon, 1997; Nikkah et al., 1995). Therefore, the adult CNS environment is likely to cause more frequent growth cone collapse of transplanted neurons, increasing the time needed for the axon to reach its target. Most transplanted neuronal phenotypes only persist in a growing state for a limited time. In fetal allotransplantation to the adult host brain, this time is usually too short for the axon to reach distant targets. Xenotransplantation experiments using donor species with slower embryonic development than the host, e.g. pig(Isacson et al., 1995) or human(Wictorin et al. 1990), transplanted to adult rats, show that the longer "time window" for outgrowth in this paradigm allows the axons to reach long distance targets. One frequently used explanation for this is that axons from the xenotransplants could be less responsive to growth inhibitory molecules expressed in the host brain. This is certainly possible, but less likely since we have preliminary observations that the growth from xenotransplants in the adult is relatively impeded compared to growth in developing (P7) brain(Isacson

and Deacon, 1997; Isacson et al., 1995), indicating that growth inhibition seen in allotransplants is also present in xenotransplants. Also, mouse fetal tissue xenotransplantated to rat striatum does not show the increase in axonal growth relative to rat donor tissue, as seen in xenotransplantation of human tissue(Isacson and Deacon, 1997; Isacson et al., 1995).

An intrinsic program for axonal outgrowth could be correlated with a low responsiveness to inhibitory signals from the environment. As the neuron matures, the upregulation of receptors for growth inhibitors would slow down growth. The ability for long distance axonal growth from xenotransplants could therefore result from a longer time before these receptors are expressed correlated with a longer time while certain growth promoting molecules are active within the neuron. Given that PNS neurons and some CNS neuronal phenotypes such as noradrenergic and cholinergic(Bjorklund and Stenevi, 1979; Björklund and Stenevi, 1977) retain their ability to regenerate throughout adulthood, there must be molecules and intrinsic processes that allow such confirmed axonal growth. These intrinsic properties may involve the same growth promoting molecules that are active during the short time when embryonic transplanted CNS neurons are able to extend axons despite the inhibitory properties of the adult CNN(Schwab et al., 1993).

What are the other factors involved in this transition of the host environment from growth permissive to growth inhibitory?

High levels of growth factors are present in the brain during development and correlate in time and location with enhanced growth of specific groups of neurons. For example, nerve growth factor (NGF) is highly expressed in the hippocampus and cortex during development(Kaisho et al., 1991) and likewise glial-derived neurotrophic factor (GDNF) is expressed in the developing striatum(Strömberg et al., 1993). There are

several studies showing improved innervation of tissue surrounding the graft when the graft is exposed to growth factors. Granholm and colleagues showed more extensive innervation from dopaminergic cells transplanted to the striatum after pretreatment of the fetal tissue with GDNF(Granholm et al., 1997). In addition to improved innervation of nearby target tissue, long distance growth has also been achieved using growth factors. For example, Wang et al. (Wang et al., 1996) created a track of GDNF along which axons from grafted dopaminergic neurons placed in the SN could grow to the target striatum. Furthermore, expression of growth factors may be the cause of more extensive or long distance axonal outgrowth from grafted neurons in a number of experiments using co-transplantation of embryonic tissue. Transplantation of a mixture of fetal VM and striatum into adult 6-OHDA lesioned striatum results in a larger area of dense innervation surrounding the graft(Brundin et al., 1986). When transplanting embryonic VM to the lesioned SN and creating a bridge of embryonic cortical tissue along an oblique needle track from the SN to the striatum, Dunnett and colleagues (Dunnett et al., 1989) showed that dopaminergic fibers from the graft could grow along the bridge-graft all the way to the striatum. Mendez and colleagues (Mendez et al., 1996) transplanted fetal VM cells simultaneously to the SN and the striatum of adult rats. Surprisingly, growth of dopaminergic axons from the graft in the SN to the VM graft in the striatum was observed, using retrograde labeling with flourogold. As a control, fetal cortical neurons transplanted to the striatum did not cause the homotopically placed dopaminergic neurons to grow to the striatum. A possible explanation for this long distance axonal growth could be the release of appropriate trophic factors by fetal cells, thereby stimulating long distance directional growth of axons, since axon orienting (tropic) factors are still present in the adult CNS(Isacson et al., 1995).

In summary, these results support the notion that maintained bcl-2 expression can enhance an axon's ability to grow. The down-stream effector molecules of such a

process are not determined, but could involve suppression factors involved in cell differentiation, such as p21. Other metabolic intracellular signals, such as cyclic AMP, PKA, GAPs, phosphorylated cytoskeletal protein and associated growth-cone cell machinery all dynamically interact to achieve the axonal growth.

FIGURES

Figure 1:

The method used for measuring approximate size and fiber outgrowth from the graft. Concentric circles (L1, L2, L3) were drawn from the center of the graft and divided into 8 sectors. The graft was outlined and the edge of the graft in the section was determined. The number of TH positive neurons in the section through the graft were counted, the distance from the center to the edge of the graft was measured. Segments were selected depending on graft size and shape, and the number of TH positive fibers crossing L1, L2, L3 in the selected segments was noted. The distance from L1, L2 and L3 was constant from the center of the graft in each case.

Figure 2:

Hubcl-2 staining of the adult transgenic mouse brain (a), VM cell culture (b), xenografts of E14 VM cell suspension from hubcl-2 transgenic mice into the substantia nigra of the rats (c-d). Hublc2 positive (c) and TH positive (d) neurons can be identified in the graft.

Figure 3:

The distance and the number of TH positive fiber crossings in striatal grafts of VM cells from wild type and hubcl-2 transgenic mice.

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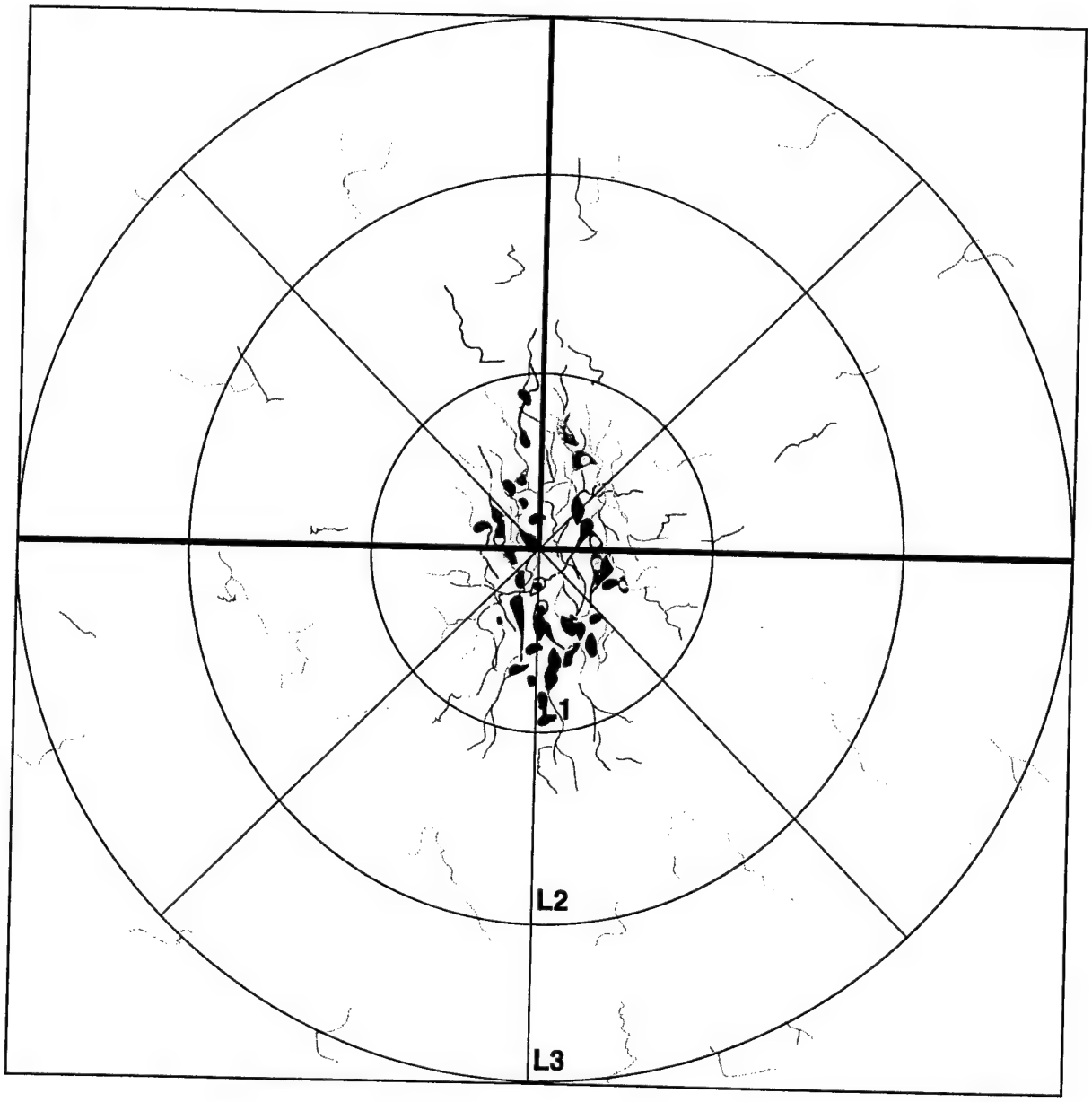
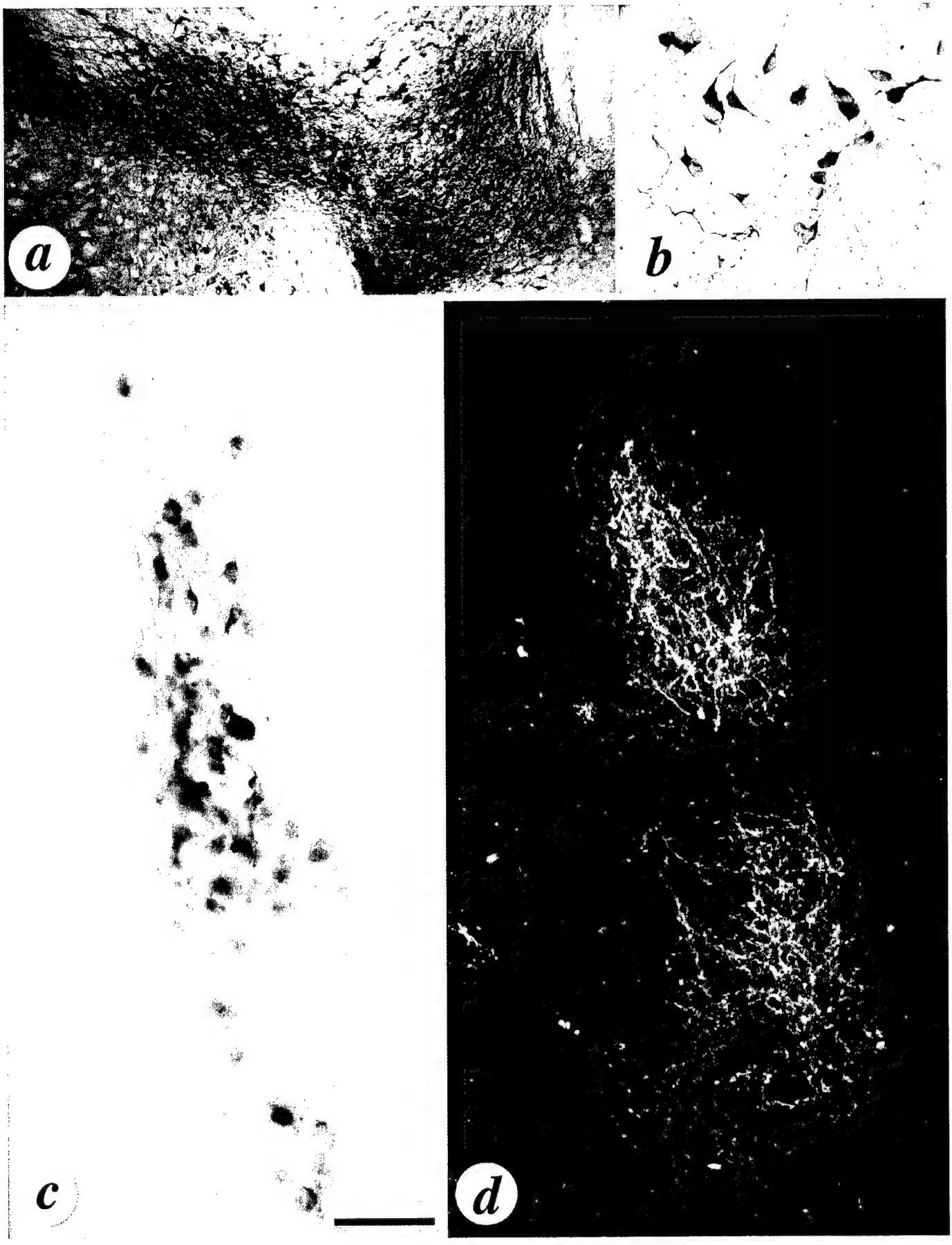
Table 1.

Groups	# of TH cells in graft section	Edge of graft from center of graft (μm)	Distance of L1 from edge (μm)	Number of fibers crossing L1	Distance of L2 from edge (μm)	Number of fibers crossing L2	Distance of L3 from edge (μm)	Number of fibers crossing L3
Bcl2 +/-								
1	38	95.03	82.97	43	262.97	28	436.97	9
2	80	115.12	59.28	33	234.88	48	417.88	65
3	12	86.91	91.09	16	270.09	8	451.09	4
4	11	82.10	94.40	24	270.30	43	420.30	69
5	13	78.80	97.70	15	280.00	13	450.60	12
Mean	**30.80**	**91.59**	**85.09**	**26.20**	**263.65**	**28.00**	**435.37**	**31.80**
SEM	**13.29**	**6.48**	**6.90**	**5.37**	**7.68**	**7.91**	**7.12**	**14.44**
Wildtype								
1	29	118.50	54.40	30	229.74	6.00	412.48	3.00
2	22	125.01	53.09	29	227.99	14.00	400.99	4.00
3	22	115.33	63.47	11	236.67	23.00	410.57	25.00
4	43	205.61	27.76	17	150.89	21.00	324.99	11.00
5	74	134.70	48.70	43	223.60	49.00	402.20	7.00
Mean	**38.00**	**139.83**	**38.38**	**26.00**	**213.78**	**22.60**	**390.25**	**10.00**
SEM	**9.78**	**16.92**	**16.71**	**5.57**	**15.86**	**7.24**	**16.47**	**4.00**


L1
L2
L3


a
b
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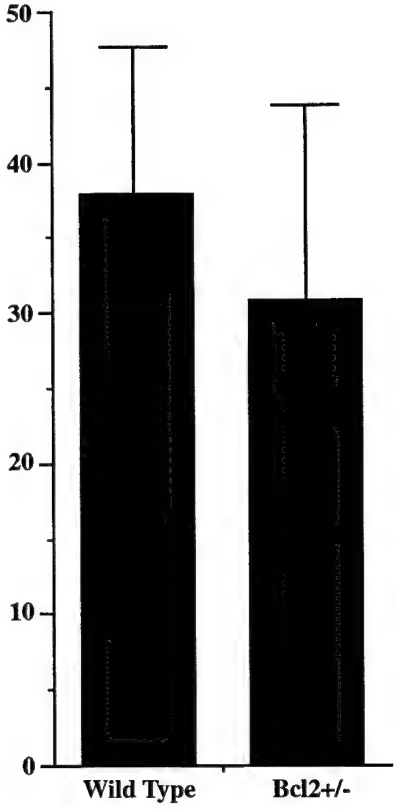
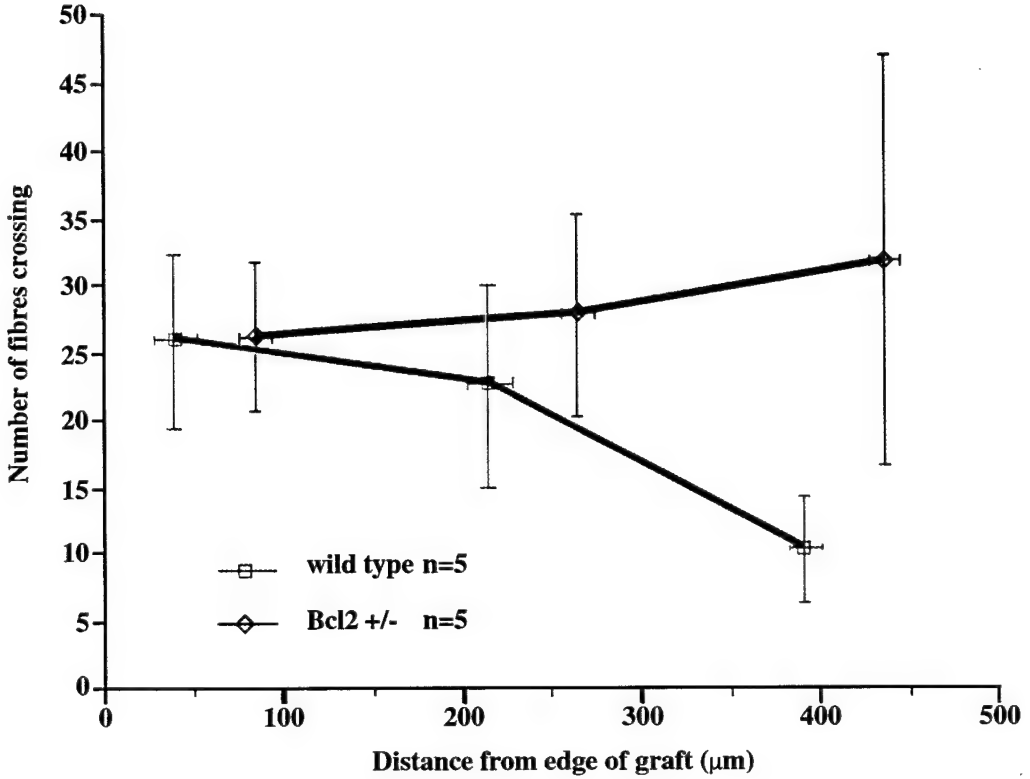
A


50
40
30
20
10
0
Wild Type
Bcl2+/-

B


Number of fibres crossing
50
45
40
35
30
25
20
15
10
5
0
0
100
200
300
400
500
Distance from edge of graft (μm)
wild type n=5
Bcl2 +/- n=5

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COMBINED APOPTOSIS AND COMPLEMENT INHIBITORS IMPROVE PORCINE NEURAL XENOTRANSPLANT SURVIVAL IN THE RAT BRAIN.

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We have undertaken a xenotransplantation study in rodents aimed at increasing cell survival of intrastriatal ventral mesencephalic (VM) grafts utilizing 1) inhibitors of cysteine-proteases, 2) the complement membrane attack complex (MAC) and 3) donor cells derived from transgenic pigs expressing human CD59 (a cell-surface molecule which blocks formation of the MAC). The experimental paradigm comprised 4 groups of 10 naïve rats. The groups received cell suspensions of pig E28 VM treated with either a) cysteine-protease inhibitors, b) MAC inhibitors, c) a combination of both or d) untreated VM. Animals also received systemic cyclosporine (CsA) one day before transplantation (30mg/kg) and then daily until sacrifice (15mg/kg). Five weeks post-transplantation, rats were perfused and their brains processed for tyrosine hydroxylase (TH) and pig neurofilament 70 kd protein (NF70) immunohistochemistry. Transplant cell survival (counts of TH-positive cells) and transplant volume (measures of NF70 graft area) were analyzed. There was a significant difference between the number of TH-positive cells in the group of combined inhibitors of cysteine-proteases/MAC versus the control group. This group also had grafts of significantly larger volumes when compared to controls. In summary, our preliminary observations in pig to rat transplants using a combination of inhibitors of cyteine-proteases and the MAC suggest better VM cell survival.

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Editorial Boards: Cell Transplantation; J. Neural Transplantation; J. Restorative Neurology and Neuroscience (section editor); Experimental Neurology, Neuroscience News, Inscight

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